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THE GENESIS OF THE CHARCOT-LEYDEN CRYSTAL IN THE EOSINOPHILIC LEUKOCYTE OF MAN*

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The formation of protein crystals within the cytoplasm of human cells of any type is actually an infrequent occurrence, the most noteworthy exceptions being the Charcot-Leyden crystals in the eosinophilic leukocytes, and the crystalloids of Reinke in the interstitial cells of the testis or hilar cells of the ovary.

The development of the first named crystals represents an uncommon fundamental intracellular process with rapid protein crystal formation that has no parallel in any other cell of the human body. Charcot-Leyden crystals have been described in the tissues in many cases of eosinophilic granuloma of bone,[1] in the sputum of patients with bronchial asthma,[2] and in stools associated with amebic dysentery.[2] The formation of these crystals is rapidly accomplished and can be observed *in vitro* in eosinophilic leukocytes within a few minutes after the application of surface-active wetting agents. Ayres and Starkey[3] studied the formation of these crystals by phase microscopy and concluded that they arose in the region of the eosinophilic granules, but saw no evidence that the granules were the source of the crystals. They also postulated, on the basis of other evidence, that the crystals were derived from the nucleus.

The present investigation is the first report of electron microscopic examination of developing crystals in eosinophils in ultrathin sections. New evidence indicates that a portion of the eosinophilic granule acts as a nidus in the genesis of the crystal and probably supplies the predominant molecular building material for crystal growth.

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Material and Methods

Blood was obtained from patients suffering from a variety of allergic disorders. The best preparations were made from a patient with severe bronchial asthma whose peripheral blood leukocyte count was 34,400 per cubic ml. with 75 per cent eosinophils. Twenty ml. of venous blood was mixed with 2.0 cc. of 3.8 per cent sodium citrate and 4.0 cc. of 6 per cent dextran. The plasma with leukocytes was then separated after sedimentation for 45 minutes at room temperature, according to the method of Klein, Eridani, Djerassi and Resnick.[4] Aliquots of 5 ml. of the plasma-leukocyte suspension were centrifuged for 5 minutes at 2,000 r.p.m. The supernatant was removed by aspiration, leaving only a dense suspension of leukocytes in a small amount of plasma. Approximately 5 to 10 mg. of Aerosol OT, dioctyl sodium sulfosuccinate (product of the American Cyanamid Company; obtainable from Fisher Scientific Company, Pittsburgh, Pennsylvania), on a glass stirring rod was added to one such concentrated aliquot, while a second similar aliquot was treated with a clean glass stirring rod as a control. Both mixtures were stirred gently at about 1 minute intervals. Within 2 minutes the aerosol-treated sample became viscid and stringy in consistency. At the end of 5 minutes both the control mixture and the aerosol-treated mixture were fixed separately in buffered osmic acid[5] with added sucrose[6] for 30 minutes. Each was then dehydrated progressively in alcohols and embedded in methacrylate. Ultrathin sections were prepared on a Porter-Blum microtome and examined in an RCA EMU 3 or an RCA EML 2 electron microscope.

Crystals were also produced by taking a similarly concentrated sterile specimen of leukocytes and incubating at 37° C. for 3 days without the addition of wetting agents. During this time interval, Charcot-Leyden crystals formed spontaneously. The specimen was then fixed and prepared for electron microscopy by the procedure outlined above.

In addition, smears of both the aerosol-treated suspension and control suspension were made, allowed to air dry, and then stained with Sudan black B according to the method of Sheehan.[7]

Results

In the control specimens the eosinophils were well preserved and showed no evidence of crystal formation (Fig. 1). The granules were intact, and the characteristic inner, densely osmiophilic bar could usually be distinguished from the outer, less dense substance. In a few cells a rare granule could be found showing a partial separation

of this outer substance from the inner bar. The eosinophils often contained one or more larger, very dense, oval bodies in the cytoplasm, representing the "basophilic bodies" described by Low and Freeman.[8] The nuclear membrane of the eosinophil was intact, and the nucleoplasm was finely dispersed.

The eosinophils from the specimens treated with Aerosol OT were markedly altered. The cytoplasmic membrane was usually indistinct or had partially disappeared. The cytoplasmic granules showed definite alterations. One such change commonly seen was a swelling of the granule leaving the central dense bar in a clear space surrounded by a fragmented circular band (Fig. 2). In other regions, the granule was swollen and contained numerous irregular clear spaces. The "basophilic bodies" were not altered. Charcot-Leyden crystals were found in all stages of development in the cytoplasm. The crystals showed a varying hexagonal or elongated dipyramidal shape, depending on the plane in which they were sectioned. The earliest forms, shown in Figures 3, 4, and 5, exhibited coalescence of several altered granules from which 3 or more sides of the crystal emerged. Large crystals, such as those shown in Figures 6 and 7, exhibited incorporation of the granules into the crystal proper. The altered granules and crystals had a similar internal appearance. Many larger crystals were perfect in shape without evidence of included granules. High magnifications of the crystals, up to 190,000, revealed an irregular separation of the crystal substance into dense, amorphous masses, between which there was a less dense space. Occasional dark ropy strands were intermingled (Fig. 8). There was no evidence of a discrete lattice or pattern of arrangement of the crystal substance.

The nucleus of the eosinophil was still distinct, but the nuclear membrane was fragmented. The nucleoplasm was aggregated into small particles or arranged in coarse strands. The eosinophils varied in the severity of the changes described above, as if the wetting agent did not affect all the cells to an equal degree. A rare eosinophil had an intact cell membrane, and granules were unaltered. No crystals were seen in these better preserved cells. Crystals were found only in those cells showing definite alterations in the granules. There was no evidence of crystal formation in the nucleus proper.

The cells from the specimen not treated with wetting agents, but incubated for 3 days, were poorly preserved; however, several features were noted. The eosinophils as individual cells could still be discerned, but granules had disappeared almost completely. Crystals were abundant, and could be seen in the cytoplasm. The nucleus was still

present but was a dense shrunken structure. There was no evidence of origin of the crystals from the nucleus.

Sudan black B stains of the air-dried smears produced a positive black to brown staining of the eosinophil granules in both the control and aerosol preparations. The granules were either a solid black or appeared as black rings with clear centers. Charcot-Leyden crystals did not stain with Sudan black. Occasionally, irregular lacunas containing a granule could be faintly discerned, usually near the center of a crystal. These intralacunar granules did not stain with Sudan black.

Discussion

The observations demonstrate that there is a marked structural alteration of the eosinophil granule as a prerequisite for crystal formation. The altered granules become enlarged and vacuolated, showing a tendency to separation of an outer cortical rim from a central core. The Sudan black stains exhibit no apparent change in staining characteristics of this outer cortical layer, although in the crystals with visible lacunas containing granules, the granules did not stain with Sudan black. It is possible that the lipid staining reaction of the granules in the crystals was blocked in some manner, or it may be that only the central protein core of the granule was incorporated into the crystal. Analysis of pure crystal samples by Buddecke, Essellier and Marti[9] revealed that they were made of a zinc-containing polypeptide from which 14 different amino acids were identified. The added fact that the crystals were always negative in reaction to lipid stains makes it probable then that the outer lipid cortex was not incorporated when the granule participated as a nidus for crystal formation.

Definite evidence is seen in Figure 6 that the granules are incorporated into the crystal and may merge imperceptibly with it. Further growth of the crystal probably does not occur through gross lumping of the granules on the original nidus but by an imperceptible molecular process. The molecular protein building material could be supplied by the other granules remaining in the cell, although other molecules from the cytoplasm or nucleus may contribute. The close similarity of the fine structure of the young crystal and the altered granules suggests that the eosinophil granule is probably the predominant source of building material. This investigation has not yielded evidence to indicate that the nucleus was an important contributor to the crystal substance. The best negative evidence was seen in the 3-day-old preparations without added aerosol. In these, practically all the granules had disappeared, crystals had formed, and yet the nucleus persisted as a shrunken dense mass. This would indicate that little or no nuclear

substance was utilized. Also, it should be noted that the "basophilic bodies" described by Low and Freeman[8] in the cytoplasm of the eosinophil are not involved in the nidus of crystal formation, and do not appear to alter significantly in the presence of aerosol.

The formation of these crystals should be of interest to the investigation of crystal growth of any type. Since the crystal can be sectioned, the stages of its development can be studied easily. It appeared that the genesis generally followed a pattern of nidus formation for the initiation of crystallization, with a subsequent uniform molecular build-up. This process appeared to be unique in that the nidus seemed to blend into the crystal imperceptibly. Ayres[3] showed that the crystal varied in its solubility, depending on its age. When freshly formed, the crystals were soluble in absolute methyl alcohol, but if allowed to stand 6 to 24 hours, the crystals were insoluble in methyl alcohol. This would indicate that the crystals were in a dynamic state of molecular arrangement for optimum stability even in their initial stage. It is possible that high resolution studies of older crystals may show a more discrete lattice or other arrangement within the crystal, although preliminary investigations have not been satisfactory because of technical difficulties.

This study, of course, does not shed light on the function of these crystals in human disease processes; it is, however, a step further in the knowledge of specific intracellular functions of the eosinophil. The eosinophil granule itself is an interesting and unique structure when examined by electron microscopy and by histochemical methods. The fact that it undergoes such a striking alteration before becoming the nidus for the formation of the crystalline structure represents a specific basic intracellular process. This would justify further attention and investigation of possible applications to the general field of cellular biophysical chemistry and understanding of the role of the eosinophil in allergic disorders.

Summary

The formation of Charcot-Leyden crystals was examined principally by electron microscopy. Ultrathin sections of human eosinophilic leukocytes treated 5 minutes with the wetting agent dioctyl sodium sulfosuccinate were prepared. The eosinophilic granules showed swelling, vacuolation, and an apparent alteration of the outer cortical layer. The altered granules were observed in process of coalescence, acting as a nidus from which the crystal emerged. The internal structures of the developing crystal and of the altered granules were quite similar, and there was evidence that the granules merged with the crystal proper. The nucleus did not appear to be a source of crystal substance.

It was proposed that the inner substance of the eosinophil granules was the nidus for and probably served as the basic protein building material for the crystal.

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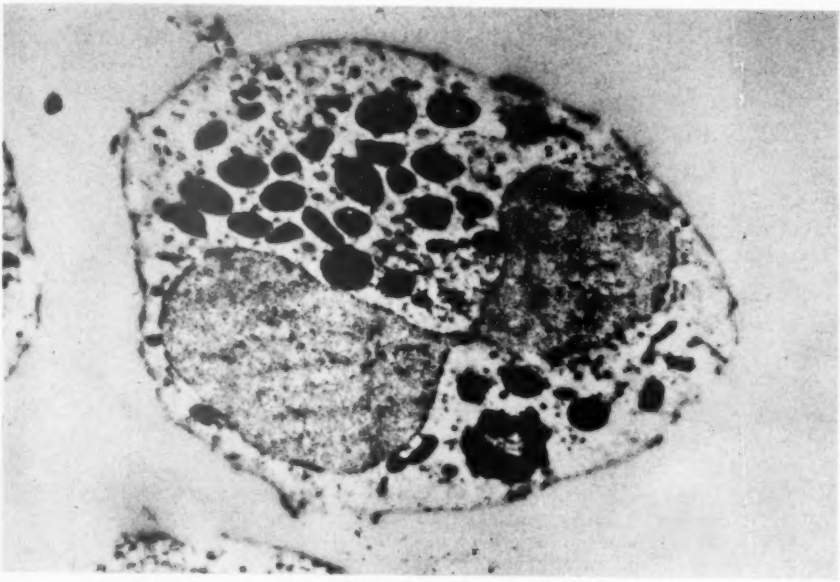
LEGENDS FOR FIGURES

FIG. 1. Untreated control eosinophilic leukocyte. There is an inner dense band visible in some of the specific granules. × 12,750.

FIG. 2. Eosinophil granules 5 minutes after addition of Aerosol OT. Some of the granules are swollen, leaving a central dense bar in a clear space surrounded by a fragmented circular band. Other granules are vacuolated. One granule appears unaltered. × 12,100.



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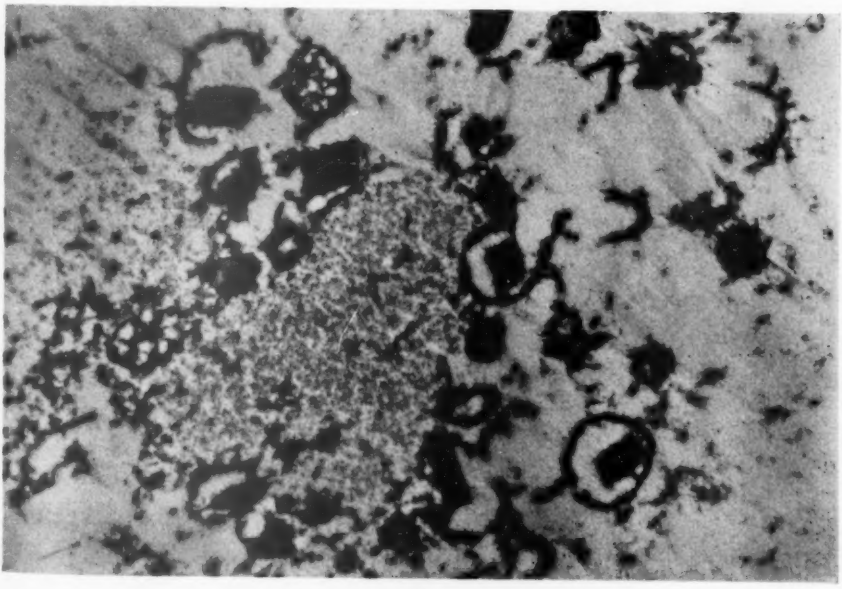

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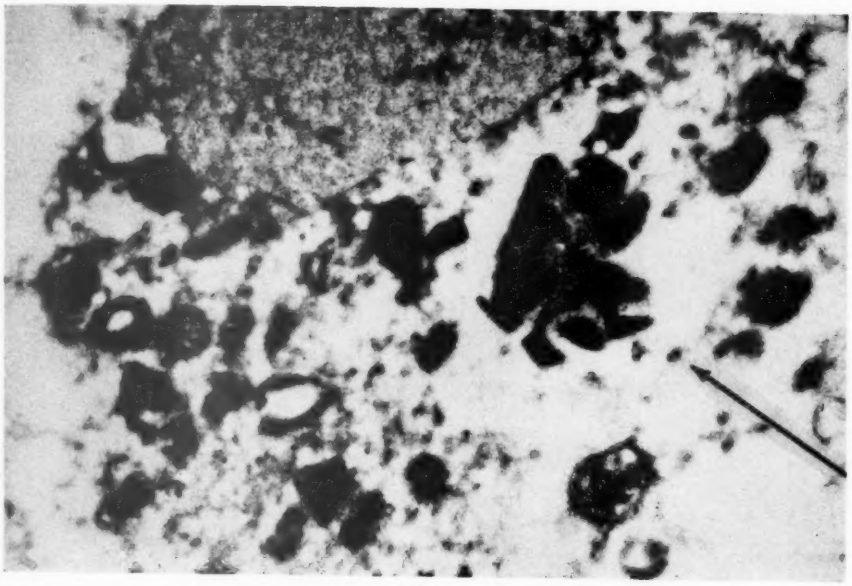
FIG. 3. Arrow points to an area of coalescence of 4 or more altered granules with emergence of angular margins of a crystal as foot-like formations. A portion of the nucleus lies in the upper mid portion of the picture; there is fragmentation of the nuclear membrane. × 15,900.

FIG. 4. In the center of the photograph an incompletely formed crystal is evident. Although most of the sides have formed, the crystal is incomplete in its upper margin and appears to be reduplicating itself on the left. The other cytoplasmic granules are altered in outline and vacuolated. × 16,900.



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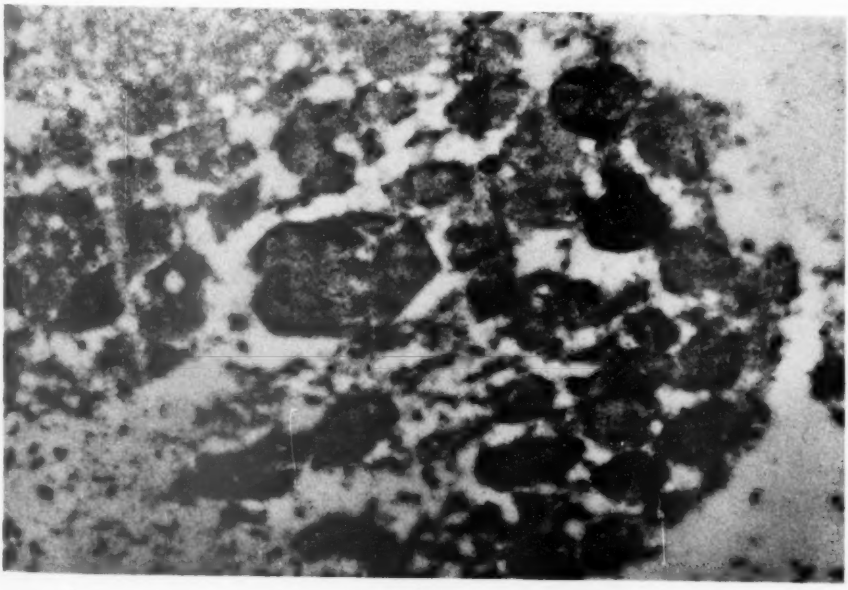

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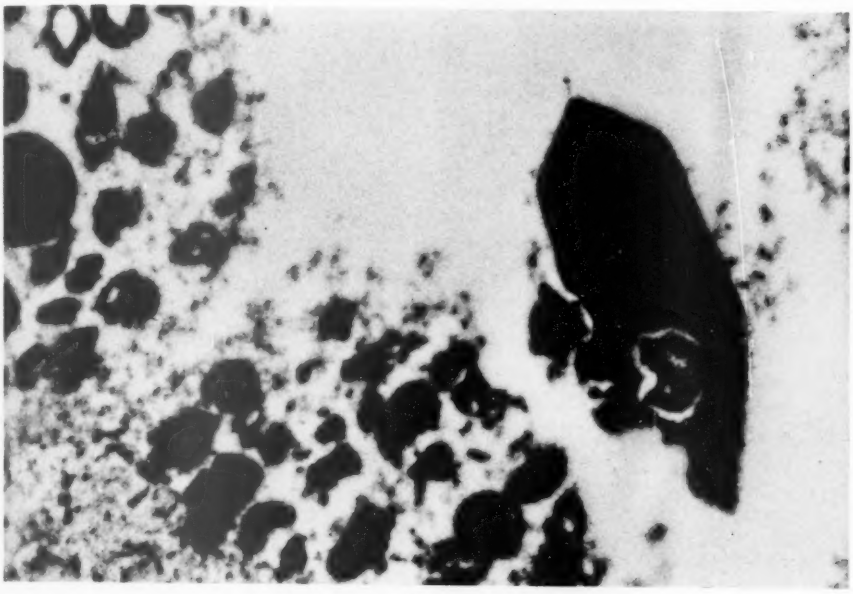
FIG. 5. A crystal developing with an irregular margin, incorporating one distinct granule. Within the crystal, above the included granule, there is a fine line forming a partial circle. This lies around what is probably another granule that has merged almost completely with the crystal. × 15,990.

FIG. 6. This crystal incorporates 3 distinct granules. × 12,750.



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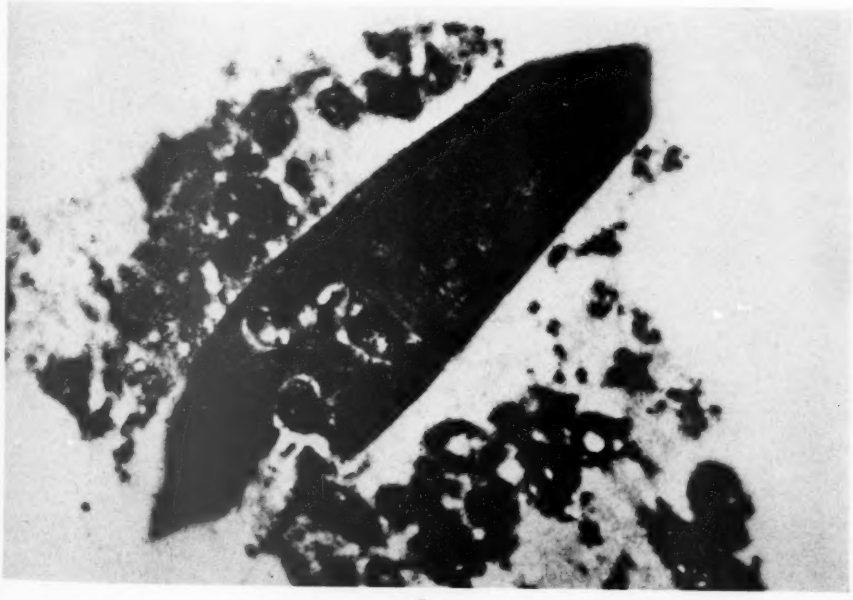

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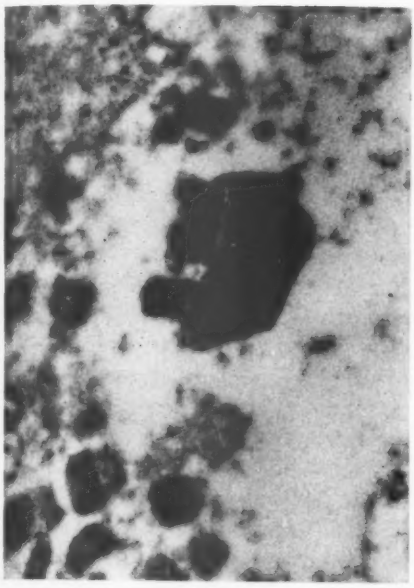
FIG. 7. Only 3 sides of this crystal have formed. The undeveloped sides contain 2 discrete granules. × 15,990.

FIG. 8. An incompletely formed crystal partially incorporating a granule. × 15,990.

FIG. 9. Eosinophilic leukocyte incubated for 3 days at 37° C.; no wetting agents were added. The nucleus persists as a vacuolated structure; all of the cytoplasmic granules have disappeared. A hexagonal crystal is present in the cytoplasm. × 12,000.

FIG. 10. Inner structure of crystal, showing dense amorphous strands irregularly separated by less dense spaces. Scattered, darker, ropy strands are manifest. × 187,200.



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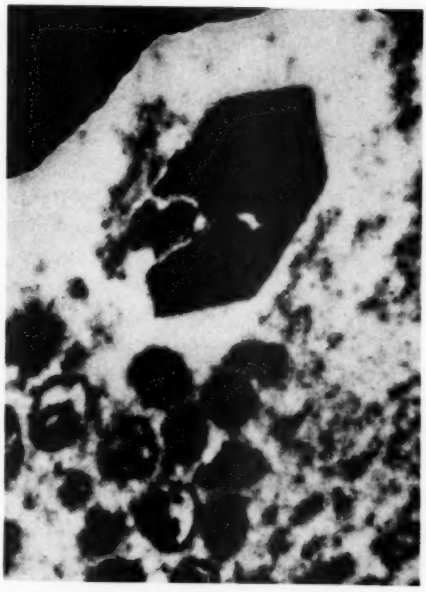

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THE PROTEIN NATURE OF ACIDOPHILIC CRYSTALLINE INTRANUCLEAR INCLUSIONS IN THE LIVER AND KIDNEY OF DOGS*

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Thompson, Cook, and Hoey[1] have recently reported the histochemical investigation of acidophilic, crystalline intranuclear inclusions (ACN) in the liver and kidney of 13 dogs. It was demonstrated that the inclusions were not composed of any of the following substances: hemoglobin or any of its iron-containing derivatives, minerals, lipids, deoxyribonucleic acid, ribonucleic acid, cholesterol, glycogen, mucin, mucopolysaccharides, polysaccharides, glycoproteins or glycolipids. The possible role of pyrimidines, purines and purine end products in the formation of the inclusions was discounted because of their insolubility in alkali, mineral acid or organic solvents. It was postulated that ACN inclusions were formed as a result of altered permeability of the nuclear membrane, which favored intranuclear retention of protein at periods of intensive protein synthesis in the nucleus. The study, herein reported, was undertaken to determine if protein was a component of the inclusions.

MATERIAL AND METHODS

The tissues employed in this investigation were procured from paraffin-embedded sections of formalin or alcohol fixed tissues from 9 of 13 dogs used in the previous study (cases 5 through 13).[1]

Serial sections, 7 to 8 μ in thickness, were prepared from the paraffin blocks in each case.

The theory of staining designed to demonstrate protein groups in tissue sections has been reported by Danielli.[2] He also reported methods for using the tetrazotized benzidine stain and suggested the application of agents to block specific groups, allowing the demonstration of other groups by staining with dinitrofluorobenzene (DNFB).[3] However, the staining procedures for DNFB were developed in this laboratory and are given below.

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Tetrazotized benzidine was used according to the procedure given by Lillie.[4] Benzoyl chloride was utilized at a concentration of 10 per cent (v/v) in pyridine. Treatment in this reagent for one hour was found sufficient to give complete blockage of the DNFB stain.

The DNFB reagent was adapted from Sanger.[5] A solution of 1 gm. of $NaHCO_3$ in 15 ml. of water was mixed with a solution of .84 ml. of DNFB in 30 ml. of ethanol. The resulting DNFB solution, saturated with $NaHCO_3$, was used at room temperature. Excess bicarbonate was allowed to settle, but was not filtered off. Tissues were regularly stained for 30 minutes. To couple the dinitrophenol derivative to a naphthol, and to enhance the color development, the nitro group was reduced with saturated stannous chloride ($SnCl_2$) in 1 N HCl for 10 minutes at room temperature. The amino group produced was diazotized in a nitrous acid solution (50 ml. of water, 1.25 ml. of concentrated HCl, and .5 gm. $NaNO_2$) for 5 minutes at ice bath temperature. This diazotized derivative was then coupled with betanaphthol, or H-acid [8-amino-1-naphthol-3, 6-disulfonic acid, monosodium salt (procured from Eastman Kodak Company, Organic Chemical Division, Rochester, New York)], according to the procedure of Danielli, as given by Lillie.[4]

According to Danielli,[2] DNFB reacts with the tyrosine groups, amino groups, and sulfhydryl groups of proteins. These groups can be blocked by diazotized p-nitroaniline, nitrous acid, and hydrogen peroxide, respectively.[2] In this work, tyrosine was blocked with diazotized aniline. The diazoaniline was prepared as a stock solution. One hundred ml. of water, 40 gm. of ice, and 3.5 ml. of concentrated HCl were mixed and cooled to 0 to 4° C. in an ice bath. After cooling, 1.38 gm. of $NaNO_2$ were dissolved in the solution, and finally 2.6 gm. of aniline hydrochloride were added with stirring. The stock solution was stored in the refrigerator, and diluted 1:30 with .1 N veronal buffer, pH 9, just prior to use. The sections were treated at ice bath temperature for 30 minutes. The procedure for blocking the amino groups in a tissue section was similar to that used in diazotizing the amino group formed from the dinitrophenol derivative, with the exception that a 15-minute exposure was used to assure complete blockade.

Sulhydryl groups were blocked by treatment with .001 M H_2O_2 at room temperature for 15 minutes.

In the procedures for the specific demonstration of amino, tyrosyl, or sulfhydryl groups, two groups were blocked as above; the section was stained with dinitrofluorobenzene, and this derivative was coupled with H-acid. The stain produced was attributed to the unblocked group.

Staining Schedules

The staining procedure for the demonstration of disulfide linkages is given below. The procedure includes the DNFB staining schedule and coupling with a naphthol (steps 5 through 13).

1. Bring slide to absolute ethanol, remove, and air dry.
2. Place immediately in benzoyl chloride reagent at room temperature for one hour.
3. Rinse with absolute ethanol, take to equilibrium with water, and wash in running water for 5 minutes.
4. Place section in 1 N HCN solution in hood at room temperature for 15 minutes.
5. Wash in running water for 5 minutes.
6. Place in DNFB solution at room temperature for 30 minutes.
7. Rinse in absolute ethanol for 1 minute; ether, 30 seconds; bring back through ethanol to water; wash in running water for 1 minute.
8. Place in saturated stannous chloride in 1 N HCl at room temperature for 10 minutes.
9. Wash in running water for 5 minutes.
10. Place in nitrous acid solution at ice bath temperature for 5 minutes.
11. Equilibrate section in 2 water baths and 3 buffer baths (.1 N veronal, pH 9) at ice bath temperature for 1 minute each.
12. Place in .05 per cent betanaphthol or H-acid solution in .1 N veronal buffer, pH 9, at ice bath temperature for 15 minutes.
13. Wash section in running water for 5 minutes, counterstain with hematoxylin and mount in standard mounting medium.

For the specific demonstration of amino, tyrosyl, or sulfhydryl groups, the groups were blocked individually. By blocking two of the reactive groups, the third could be demonstrated. In order to provide a color comparison, sections which had been treated with all 3 blocking agents and sections which had not been blocked were run. The following schedule includes all 3 blocking agents. To demonstrate a particular group, the blocking reaction for that group was omitted from the procedure.

1. Bring section to equilibrium with water at ice bath temperature.
2. Place section in nitrous acid solution at ice bath temperature for 15 minutes.
3. Rinse section in cold running tap water for 5 minutes, and equilibrate in .1 N veronal buffer, pH 9 at 0 to 4° C.
4. Place section in diazoaniline solution at ice bath temperature for 30 minutes.
5. Wash in cold running tap water for 5 minutes.
6. Place section in .001 M H_2O_2 solution at room temperature for 15 minutes.
7. Wash in running tap water for 5 minutes and proceed with the DNFB staining schedule given in steps 6 through 13 above.

In addition to the procedures given above, liver and kidney sections from cases 5, 6, 10, 11, and 13[1] were subjected to the procedures of Barrnett and Seligman[6-8] for sulfhydryl and disulfide groups, utilizing the 2, 2′-dihydroxy-6, 6′ dinaphthyl disulfide (DDD) stain. Prior to staining for sulfhydryl groups, the deparaffined sections were washed with running tap water for 1½ hours to liberate the sulfhydryl groups.[9] In the disulfide procedure, benzoyl chloride was used to block the sulfhydryl groups.

Results

The results obtained with each staining procedure were sufficiently uniform that the reaction of the acidophilic, crystalline intranuclear (ACN) inclusions can be reported without reference to the tissue or case in which they occurred (Table I). The color characteristics of each stain were as follows:

1. Tetrazotized benzidine. The color range of the ACN inclusions was red to orange, that of the nucleoplasm was blue to violet, and the cytoplasm was orange to orange-brown (Fig. 1).

2. Benzoyl chloride and tetrazotized benzidine. The ACN inclusions were unstained, the nucleoplasm was red to blue, and the cytoplasm was unstained to faint brown (Fig. 2).

Table I

Staining Characteristics of ACN Inclusions

Protein stain	Reaction of inclusions
Tetrazotized benzidine	Positive
Benzoyl chloride and tetrazotized benzidine	Negative
Dinitrofluorobenzene	Positive
Tyrosyl — DNFB	Positive
Amino — DNFB	Weakly positive
Sulfhydryl — DNFB	Positive
Disulfide — DNFB	Negative to weakly positive
Sulfhydryl — DDD	Positive
Disulfide — DDD	Negative

3. Dinitrofluorobenzene (DNFB). The color range of the ACN inclusions was red-brown to mulberry, that of the nucleoplasm was blue to violet, and the cytoplasm was brown (Fig. 3).

4. Tyrosyl groups (DNFB). The color range of the ACN inclusions was indigo to brown, that of the nucleoplasm was purple, and the cytoplasm was light red to tan (Fig. 4).

5. Amino groups (DNFB.) The color range of the ACN inclusions was faint yellow to tan, that of the nucleoplasm was blue to purple, and the cytoplasm was yellow to tan (Fig. 5).

6. Sulfhydryl groups (DFNB). The color range of the ACN inclusions was orange to carmine, that of the nucleoplasm was purple, and the cytoplasm was carmine (Figs. 6 and 7).

7. Disulfide groups (DFNB). The ACN inclusions were usually unstained but occasionally showed a bluish tinge; the nucleoplasm was violet, and the cytoplasm was yellow to tan.

8. Sulfhydryl groups (DDD). The ACN inclusions, nucleoplasm

and cytoplasm were all violet, but the inclusions were more intensely stained (Fig. 8).

9. Disulfide groups (DDD). The ACN inclusions were unstained, the nucleoplasm was faint blue, and the cytoplasm was light yellow.

Discussion

The evidence presented by Thompson, Cook and Hoey[1] indicates that the ACN inclusions do not contain demonstrable carbohydrates, lipids, minerals, or nucleic acids. The results obtained in the present study tend to confirm the previously postulated protein nature of these inclusions. It is thought that the specific reactive groups of protein, which must be in an available form prior to staining,[10] are not appreciably masked by the alcohol or formalin[9,11] fixatives used.

The positive staining with tetrazotized benzidine indicates that the ACN inclusions contain protein or nucleic acid.[3] When tissue sections are blocked by benzoyl chloride and subsequently stained by the same method, only the nucleic acid remains reactive.[4] The ACN inclusions were not stained following treatment with benzoyl chloride. This indicates the absence of nucleic acid[1] and shows that the positive stain of the ACN inclusions with tetrazotized benzidine alone is due to reactive groups normally found in protein.

Most viral inclusions which have been studied histochemically have exhibited positive reactions to stains for nucleic acids.[12,13] The absence of nucleic acid in ACN inclusions as demonstrated in this study greatly lessens the possibility that viruses play a role in the formation of the inclusions.

The protein nature of the ACN inclusions was directly demonstrated by the DNFB stain, which stains tyrosyl, amino and sulfhydryl groups, but not nucleic acids.[2] The specific stains for tyrosyl, amino, and sulfhydryl groups using the DNFB method gave a well defined positive reaction for tyrosine and sulfhydryl, but a weak reaction for amino groups. This was only a qualitative demonstration of these groups, and did not necessarily reflect their relative abundance. The existence of sulfhydryl groups in the inclusions was substantiated by the positive reaction to the DDD stain; this is reported to be a specific reaction.[10]

The ACN inclusions did not stain with the DDD or DNFB disulfide procedures, except for occasional weak positive reactions following DNFB. Because of the specificity of the DDD method, it is probable that the inclusions contain no disulfides or that the concentration of these is too low to be demonstrable. The occasional weak positive stain obtained with the DNFB procedure was probably due to incomplete

blockade of some amino or tyrosyl groups. On the basis of these results, it is not possible to eliminate disulfide linkages as constituents of the ACN inclusions with certainty. However, the presence of these linkages is consistent with the probable protein nature of the inclusions and with the existence of sulfhydryl groups.

The demonstration of tyrosine, sulfhydryl groups (cysteine), amino groups (lysine, glutamine or terminal amino), and possibly disulfide linkages (cystine) in the ACN inclusions indicated that protein or polypeptides were present. The physiologic mechanism responsible for the formation of these crystalline inclusions is unknown; it may involve altered permeability of the nuclear membrane or excess protein or polypeptide synthesis within the nucleus. It is possible that the ACN inclusions were formed post mortem by the aggregation of protein-like constituents of the nucleus in cells which had a high rate of protein synthesis just prior to death; but this is not likely in view of the extreme distention of the nuclear membrane in some instances, as well as the margination of chromatin. The histologic appearance is that of a process which occurred *in vivo*.

The conclusions which are drawn from this study are that the ACN inclusions contain reactive groups normally associated with the presence of protein or polypeptides. Other compounds normally occurring in the liver and kidney epithelial cells and capable of detection by the histochemical methods used in this and the previous study were not demonstrable.

Summary

Acidophilic, crystalline intranuclear inclusions observed within the epithelial cells of the liver and kidneys of 9 dogs were shown to contain reactive groups normally associated with the presence of proteins or polypeptides. Staining schedules are given for the demonstration of specific protein groups in formalin and alcohol fixed, paraffin-embedded tissue sections, utilizing a dinitrofluorobenzene technique.

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[*Illustrations follow*]

LEGENDS FOR FIGURES*

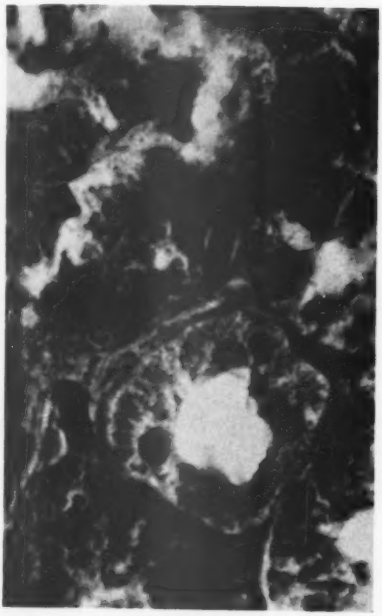
FIG. 1. Kidney of a dog. Single ACN inclusion in a renal tubular epithelial cell. Section stained with tetrazotized benzidine coupled with betanaphthol to demonstrate tyrosine, histidine, tryptophan and the purines and pyrimidines of nucleic acids. × 1000.

FIG. 2. Kidney of a dog. Two unstained ACN inclusions in renal tubular epithelial cells. Section treated by benzoyl chloride to block tyrosine, histidine and tryptophan and stained with tetrazotized benzidine coupled with betanaphthol. × 1000.

FIG. 3. Kidney of a dog. Single ACN inclusion in a renal tubular epithelial cell, stained with dinitrofluorobenzene coupled with H-acid to demonstrate tyrosyl, amino and sulfhydryl groups. × 1000.

FIG. 4. Liver of a dog. Single ACN inclusion in hepatic epithelial cell. Section treated by hydrogen peroxide to block sulfhydryl groups and nitrous acid to block amino groups. The dinitrofluorobenzene stain, coupled with H-acid was employed to demonstrate tyrosyl groups. × 1000.

* United States Army photographs, taken by Mr. William Hummel of the Pathology Service, Fitzsimons Army Hospital, Denver, Colo.



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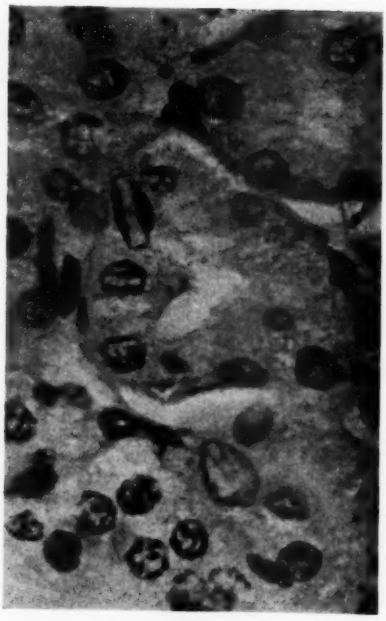

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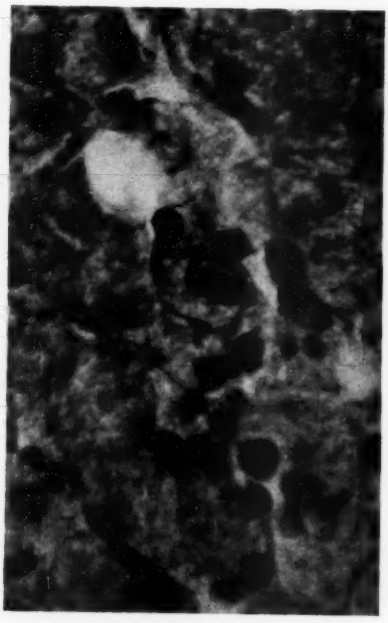

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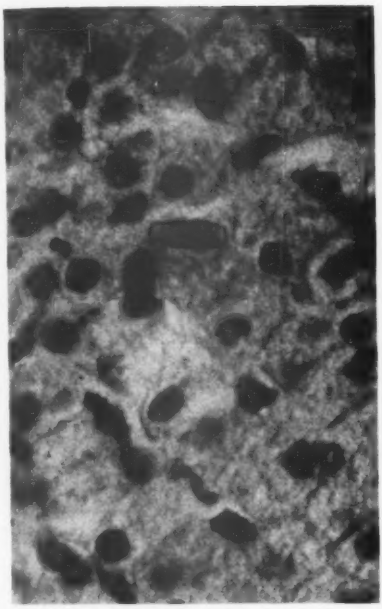
FIG. 5. Liver of a dog. Single ACN inclusion in a hepatic epithelial cell. Section treated by hydrogen peroxide to block sulfhydryl groups and diazoaniline to block tyrosyl groups. The dinitrofluorobenzene stain coupled with H-acid was employed to demonstrate the presence of amino groups. × 1000.

FIGS. 6 and 7. Liver and kidney of a dog. Single ACN inclusions are shown in epithelial cells of each organ. Sections treated by diazoaniline to block tyrosyl groups and nitrous acid to block amino groups. The dinitrofluorobenzene stain coupled with H-acid was employed to demonstrate the presence of sulfhydryl groups. × 1000.

FIG. 8. Kidney of dog. Single ACN inclusion in a renal tubular epithelial cell. The presence of sulfhydryl groups is demonstrated by the 2, 2′-dehydroxy-6, 6′ dinaphthyl disulfide stain. × 1000.



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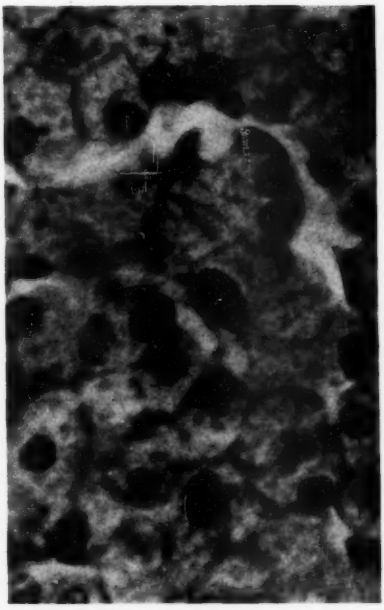

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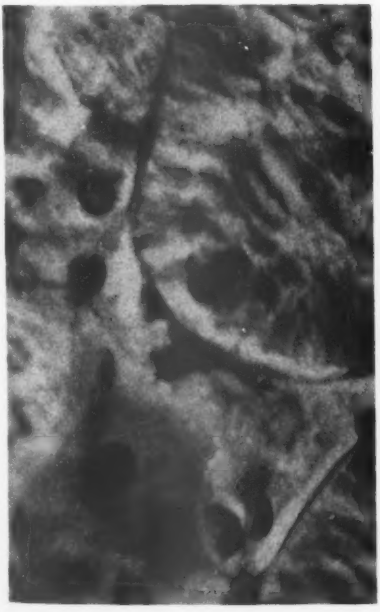

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CYTOPLASMIC INCLUSIONS IN GANGLION CELLS ASSOCIATED WITH PARKINSONIAN STATES

A Neurocellular Change Studied in 53 Cases and 206 Controls *

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Among the less well known forms of neurocellular degeneration is the occasional occurrence of discrete, nongranular, polychromatophilic cytoplasmic bodies in large and medium sized neurons. When present, they are usually encountered in the pigmented cells of the substantia nigra and other "neuromelanin"-containing ganglion cells of the brain stem. Since Lewy's[1,2] original descriptions in 1912 of these structures in the *ansa lenticularis* and the dorsal vagal nucleus, they have been subjected to only infrequent and intermittent investigation. To Trétiakoff[3] must go the credit for describing these bodies in their most common site of occurrence, the substantia nigra, and the demonstration of a possible relationship to paralysis agitans. Hassler[4] stated that he had never seen the bodies in the normal senile brain, while Klaue[5] questioned their specificity in idiopathic parkinsonism. Greenfield and Bosanquet[6] in 1953 presented the first adequate description of the Lewy body in English, considering it as quite specific for paralysis agitans when it occurred in association with degeneration of certain cell groups of the substantia nigra. The present study has the twofold objective of determining the relationship of these bodies to the various forms of parkinsonism, and of clarifying further the nature of the cytoplasmic alteration.

The terms "paralysis agitans" and "idiopathic parkinsonism" will be used interchangeably in this report. Along with "postencephalitic parkinsonism," as defined below, they are designations implying a specific etiologic factor or lack of knowledge thereof. "Parkinsonian states" and "parkinsonism" (without modifying adjective) are employed as synonymous generic terms referring to a clinical constellation without reference to etiology or pathologic lesions.

Selection of Cases

Group A

This group consisted of 53 patients with parkinsonism of all types —all that had had complete necropsy examination during the periods

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1948 to 1957 at the Jewish Chronic Disease Hospital and 1954 to 1957 at the Kings County Hospital. Patients exhibiting other extrapyramidal syndromes were excluded. Subdivision into 3 categories by detailed review of the clinical records, according to the criteria established, was carried out independently of the examination for Lewy bodies.

Postencephalitic Parkinsonism (9 cases). Twenty-two cases in group A were considered clinically to be of postencephalitic origin, but only 9 of these met the criteria cited below. No patient was retained in this category in the absence of the following features, considered valid indications of encephalitis: (1) The episode occurred during the period 1918 to 1930. (2) At least 3 of the following observations were specifically recorded: (a) fever; (b) somnolence; (c) disturbance of sleep pattern; (d) oculogyric crises; (e) other oculomotor signs, e.g., history of diplopia, etc.; and (f) other evidence of diffuse central nervous system disorder.

In addition to the above, at least 3 of the following criteria were required: (a) oculogyric crises subsequent to the acute episode; (b) evidence of other oculomotor damage of nontransient nature, persisting beyond or developing after the encephalitis; (c) persistent sleep disturbance; (d) onset of parkinsonian symptoms (at least two of the following: tremor, rigidity, masklike facies, weakness, easy fatiguability, weakness of voice) within 10 years of the onset of the encephalitic episode; (e) rapid progression early in the course, with dominance of rigidity within 5 years of onset; (f) sialorrhea; (g) appearance of tics subsequent to the episode of encephalitis. Age of onset was not considered a criterion since this constituted one of the variables under study.

Unclassified Parkinsonian States (15 cases). This subdivision included the 13 cases considered to be postencephalitic on clinical grounds but which could not be included in the postencephalitic group as previously defined. Two additional cases with the parkinsonian state, in which an adequate history was lacking or in which demise followed too rapidly for adequate classification, were also included.

Paralysis Agitans (29 cases). This subgroup consisted of those patients in whom the onset of parkinsonism was insidious, progression was gradual, initial manifestations were often localized, but eventual involvement of all extremities was recorded. All the patients were considered by the clinical observers to have the idiopathic type of disorder, i.e., true paralysis agitans.

Group B, Controls

This group consisted of 206 consecutive adults who had had complete necropsy examinations in 1957. These remained after detailed independent review of the clinical records had eliminated all possible cases of parkinsonism, or those in which information was insufficient to allow adequate clinical evaluation.

Matched Controls (106 cases). All members of group B were listed in numerical order of necropsy numbers with indications of age, sex, and race, but without reference to or knowledge of the presence of Lewy bodies. Each case of parkinsonism was taken in order and matched with the first two listed cases of the same age, sex, and race in the control group. In those instances in which this was not possible, the case was held until succeeding cases were matched. The unmatched balances were then successively matched with an allowable age range of ± 1, then ± 2, and finally ± 3 years. In no instance was the requirement for corresponding race or sex relaxed, and it was unnecessary to exceed the 3-year range.

TABLE I

Age and Duration of Illness in 53 Examples of Parkinsonian States

Parkinsonian states	Mean age		Mean duration of illness		
	At onset	At death	"Positive"*	"Negative"	Total
Postencephalitic	29.4 ± 3.6†	50.5 ± 2.9			28.5 ± 3.1
Idiopathic	54.6 ± 1.9	65.5 ± 1.3			10.3 ± 1.4
Unclassified	41.4 ± 3.3	61.6 ± 2.2			21.6 ± 2.5
All parkinsonian states	46.9 ± 1.8	61.6 ± 1.2	14.5 ± 1.4	22.0 ± 2.8	16.6 ± 1.3

* Instances in which ganglionic intracytoplasmic bodies were encountered.
† The values given are the arithmetic means ± the standard error.

Unmatched Controls. The control cases remaining following the matching procedure were divided into two groups using the mean age of the parkinsonian state group as the arbitrary point of division (61.6 years); 41 cases were younger and 59 were older than this age.

METHODS

Morphologic Investigation

Sections from all cases, study and control, were screened for the presence of Lewy bodies in a single slide, 5 to 7 μ in thickness, and stained with hematoxylin and eosin. When the slide represented only a midbrain hemisection, 2 such were screened. On each slide an area

outlined with India ink, encompassing the entire substantia nigra and a broad adjacent zone, was subjected to a systematic cell-by-cell examination at a magnification of 440 to 660 times. Sections of cortex, thalamus, caudate nucleus, putamen, globus pallidus, pons (including locus caeruleus), cerebellum (roof nuclei as well as cortical structures), and medulla (including dorsal vagal nucleus and inferior olive) were examined in an identical manner in 14 selected cases of parkinsonian states and in 4 of the controls in which the bodies were encountered on examination of the substantia nigra.

The criteria established for the Lewy body were: (a) intracytoplasmic location within a cell identifiable as a neuron (to be considered a neuron, a cell was required to contain either a typical neuronal nucleus, or identifiable Nissl substance, and preferably both; the presence of "neuromelanin" pigment alone was not considered sufficient); (b) nongranularity; (c) polychromatophilia; and (d) either a surrounding halo or clear zone or a central density visible under phase contrast microscopy. Adherence to all criteria was required in order to exclude such structures as extruded "hypertrophic nucleoli" (Wolf-Orton "inclusions"[7]), as well as conglomerated eosinophilic "protomelanin" granules. An arbitrary grading system was employed as follows: If 1 to 5 Lewy bodies were present in the entire section or in 2 hemisections, the section was rated as 1+; 6 to 20 bodies, as 2+; and greater than 20, as 3+.

The sections in both the parkinsonian and control series were taken from various levels, but the large majority from both groups represented the caudal half of the extent of the substantia nigra. Following screening, systematic scanning at a magnification of 100 or 150 times was performed to form an impression as to the relative number of pigmented cells.

Histochemical Investigation

The following staining procedures were carried out simultaneously on selected sections of all categories of cases in which bodies were encountered: Periodic acid-Schiff[8a]; Feulgen[8b]; methyl green-pyronin (2 methods[9,10]); azure B[11]; acridine orange fluorescence[12] at pH 4.8, both with and without ribonuclease[11]; 10 per cent perchloric[13] and 5 per cent trichloroacetic acid[14] pretreatment; tests for lead[15] and iron[8c]; chromotrope aniline blue[16]; trichrome[8d] on zenkerized sections; Sudan IV in isopropyl alcohol; Sudan black B in propylene glycol; phosphin 3R fluorescence[17]; and acid-fast stains.[18]

Results

The relationships between the parkinsonian states on the one hand and the ages at onset, at death, and the duration of illness, on the other, are summarized in Table I. There were 29 males and 24 females in the group with parkinsonian states. The mean brain weights (± the standard errors) in grams were: for all patients with parkinsonian states, 1,234 ± 27; for males, 1,285 ± 26; for females, 1,145 ± 30; for the patients with the postencephalitic variety, 1,338 ± 62; and for the patients with idiopathic disorder, 1,238 ± 26.

Encephalomalacia was noted in 7 cases, 5 of which were in the idiopathic group and one each in the unclassified and postencephalitic groups; none showed involvement of the substantia nigra. Gross atrophy of the globus pallidus was observed in 9 of 25 cases (36 per cent) where this structure was described in sufficient detail. Conclusive microscopic evidence of pallidal atrophy or degeneration was noted in 30 of 34 cases (88 per cent). No valid quantitative estimate of the degree of either atherosclerosis or of arteriolar sclerosis was possible.

In 45 of the 53 cases (85 per cent) of parkinsonism, specific mention was made of the gross appearance of the substantia nigra and in all of these some degree of pigment loss was described. However, no quantitative criterion regarding this change could be established from the descriptions of several different prosectors.

In the matched control group, 21 cases (19.8 per cent) exhibited grossly evident or microscopically significant lesions, but were without clinically recorded signs or symptoms during life which could be ascribed to the central nervous system. Thirty-five cases (33 per cent) were without morphologic or clinical evidence of central nervous system involvement while 42 cases (39.6 per cent) exhibited both a significant cerebral lesion and clinically manifest evidence of neurologic disorder. In 8 cases there were no morphologic lesions, but some clinical evidence suggestive of neurologic dysfunction was manifest.

The commonest lesion encountered in the control group was encephalomalacia. This was present in 44 cases (41.5 per cent) or in 69.8 per cent of all cases with significant morphologic alteration in the brain or cord. Other conditions present were suppurative meningitis, tuberculous meningitis, brain tumors (astrocytomas, grades II and IV, and meningioma), brain abscess and, in a single instance, cryptococcal meningitis.

In none of the cases in either of the control groups was any specific mention made of the disproportionate atrophy of the globus pallidus or depigmentation of the substantia nigra.

Histologic Nature of the Ganglionic Intracytoplasmic Bodies

The occurence of Lewy bodies, ganglionic intracytoplasmic bodies (GIB), in the substantia nigra is summarized in Table II. Cases showing the GIB are designated as "positive." They were encountered in all groups of parkinsonian states and in cases with both short and long clinical duration. In the matched control group, 2 of the "positive" cases were without morphologic or clinical evidence of central nervous system disease, 1 "positive" occurred in a case of tuberculous meningitis, while in 3 there were coincident encephalomacic processes of varying degrees and locations. No morphologic or histochemical differences were observed among the GIB encountered in the various subgroups of parkinsonism, or in the "positive" controls.

TABLE II

Incidence of Ganglionic Intracytoplasmic (Lewy) Bodies in the Substantia Nigra

Group	Total cases	Grading*			Total "positive"	Per cent "positive"
		1+	2+	3+		
Postencephalitic	9†	3	1	0	4	44.4
Idiopathic	29	12	6	7	25	86.2
Unclassified	15	8	2	1	11	73.3
All parkinsonian states	53	23	9	8	40	75.5
Matched controls	106	4	2	0	6	5.7
Unmatched controls:						
Older than 61.1 years	59	2	0	2	4	6.8
Younger than 61.1 years	41	0	0	0	0	0
All controls	206	6	2	2	10	4.9

* See text for grading system.

† One of these cases, here considered negative, had a single Lewy body in the locus caeruleus.

Lewy bodies were usually circular—though occasionally elongated—discrete, and without demonstrable granularity. They were frequently multiple and almost invariably surrounded by a halo or clear zone. The latter was occasionally traversed by a few thin threadlike strands which passed from the cytoplasm to the surface of the body proper. These were visible only with maximum resolution of the light microscope.

With hematoxylin and eosin staining, the body was magenta colored, and a central density could be seen only very rarely. However, with acid aniline blue staining techniques, such as the Roque[16] chromotrope

aniline blue, the bodies stained a uniform blue and fairly frequently exhibited a well defined bright red staining central density. Differential staining of the core was also observed with the Masson technique where the core was stained red. The cores were demonstrated in all varieties of the parkinsonian state as well as in the "positive" controls. The bodies did not take the periodic acid-Schiff or Feulgen stains. The Mallory lead and Perls iron stains were negative.

Azure B stained these bodies uniformly a slightly deeper blue than the very pale blue of the surrounding cytoplasm, and much less intensely than the remaining Nissl substance. This slight blue staining did not occur when preceded by treatment with ribonuclease, trichloroacetic or perchloric acids. With the methyl green-pyronin technique, the GIB were stained a faint red, while with the acridine orange procedure, they were pale red or pink when observed with ultraviolet light excitation. The latter staining reaction did not occur in sections pretreated with trichloroacetic and perchloric acids.

In every instance where a nucleus was observable in a neuron containing a GIB, the former structure was eccentric and most frequently located at the pole of the cell diametrically opposite the Lewy body (Figs. 3 and 4). Pyknosis was uncommon, and frank karyorrhexis was not encountered. Occasionally, a binucleate neuron was seen to contain the body (Fig. 8). Multiple refractile eosinophilic intranuclear structures (Wolf-Orton bodies), although frequent enough in the vicinity of GIB-containing cells, were observed to occur in the same cell as the GIB in only 3 instances.

In Lewy-body-containing neurons, the Nissl substance was sometimes absent, but more often was reduced to a thin, deeply basophilic peripheral rim (Figs. 2 to 4), while the major part of the cytoplasm was uniformly and finely granular and only very weakly basophilic. When, as usual, these cells contained pigment, the number of "neuromelanin" granules frequently appeared to be less than that in adjacent cells. There was also a distinct tendency for the remaining pigment granules to cluster around the GIB and, by contrast, to accentuate the halo, forming an almost semicircular pattern (Figs. 3 and 4). Cytoplasmic vacuolation was rarely encountered (Fig. 6). Still more rarely, vacuolation coincided in space with the halo. This was noted most frequently when phase microscopic examination or acid aniline blue staining revealed a particularly prominent or dense central core (Fig. 7). Except in those instances where the degenerative changes in the neurons were particularly advanced, satellitosis was not observed in relation to neurons containing GIB.

No objective grading system could be established with respect to the

degree of cell loss. Although in a few cases of idiopathic parkinsonism, appreciable cell loss was not demonstrated, in most of them this occurred to a variable degree. In all of the cases of postencephalitic parkinsonism the paucity of neurons in the substantia nigra was immediately apparent and was accompanied by an increase in glial cells. The occurrence of GIB in areas other than the substantia nigra is summarized in Table III.

Discussion

It is fairly generally held that the substantia nigra is more or less affected in all parkinsonian states (with the exception of the rare instance of so-called "supra nigral" parkinsonism). Greenfield[19] considered ". . . paralysis agitans . . . a systematic degeneration of a special type affecting a neuronal system whose nodal point is the substantia nigra." Hassin's[20] demonstration of greater nigral involvement in the postencephalitic form, and Hassler's[4] description of preferential zones of cell destruction which he considered characteristic of certain forms of parkinsonism, while confirming the universality of involvement of this pigmented structure, cannot in either instance be used as morphologic criteria in the classification of an individual case. Since the work of Trétriakoff,[3] all authors specifically concerned with the GIB and its relationship to parkinsonian states have agreed on its occurence in the substantia nigra in cases of paralysis

Table III

*Extra-Nigral Incidence of Ganglionic Intracytoplasmic (Lewy) Bodies in Parkinsonian States "Positive" in the Substantia Nigra**

Group	Locus caeruleus	Dorsal vagal nucleus	Cerebral cortex	Caudate nucleus	Putamen	Globus pallidus	Thalamus	Cerebellar cortex	Dentate nucleus	Inferior olive
Postencephalitic	0 (1)	0 (1)	0 (2)	0 (2)	0 (2)	0 (2)	0 (1)	0 (1)	0 (1)	0 (2)
Idiopathic	7 (7)	3 (5)	1 (4)	0 (4)	0 (4)	0 (4)	0 (3)	0 (3)	0 (2)	1 (5)
Unclassified	3 (4)	1 (3)	0 (3)	0 (1)	0 (1)	0 (1)	0 (0)	0 (0)	0 (0)	0 (3)
All parkinsonian states	10 (12)	4 (9)	1 (9)	0 (7)	0 (7)	0 (7)	0 (4)	0 (4)	0 (3)	1 (10)

* The figure in parenthesis is the number of cases screened for Lewy bodies as noted in the text. Two additional idiopathic and one postencephalitic cases, all "negative," were screened in all of the above areas with the exception of the cerebellar structures, and were "negative" with the exception of a single body encountered in the locus caeruleus in an instance of the postencephalitic state.

agitans. Its appearance in other regions in this disease is also not disputed; for example, Beheim-Schwarzbach demonstrated "Masson positive vacuoles" in the locus caeruleus.[21]

However, the present observations in cases of postencephalitic parkinsonism are at variance with the conclusions of earlier authors, with the major exception of Klaue.[5] It would appear (Table II) that one is more likely to encounter a Lewy body in a section of substantia nigra in a case of idiopathic than in postencephalitic parkinsonism. Since the work of Beheim-Schwarzbach and the present investigation indicate the occurrence of these bodies, albeit infrequently, in patients without parkinsonism, it is necessary to examine the possibility that their appearance in postencephalitic parkinsonism may be due to mere chance. Statistical analysis of the incidence data shows that this is not the case ($P<0.001$).

In examples of the postencephalitic variety, the sparse occurrence of Lewy bodies may be due to the greater severity of involvement of the substantia nigra in this condition. This has been described by Hassin, and is supported by observations in the present study as indicated by more uniform and severe neuronal loss, accompanied by glial cell increase. The infrequent appearance of GIB may also be related to factors which are readily apparent in Table I. The patients with the postencephalitic type of disorder, although they developed the disease at an earlier age and succumbed at a younger age than those with the idiopathic parkinsonian state, have had a very significantly greater duration of illness. In this connection, the well known progression of the signs and symptoms of all forms of parkinsonism (initially more rapid in the postencephalitic variety) should be considered along with the data in Table II concerning the relation between the incidence of Lewy bodies and the duration of the disease. A "positive" case (mean duration, 14.5 ± 1.4 years) is more likely to be of shorter overall duration than is a "negative" one (22.0 ± 2.8 years). If it is accepted that during the course of any form of parkinsonism there is more or less gradual but progressive degenerative loss of neurons, it is then possible that in the postencephalitic form, as a result of the greater duration, fewer neurons exist which are capable of harboring these bodies.

Scanning the substantia nigra in the control series did not reveal appreciable differences with respect to the number of neurons present in "positive" as compared to "negative" cases.

The histochemical procedures employed in this study contributed little to the characterization of the bodies. The amount of ribonucleic acid must be very small indeed since the staining reactions for it were so very weakly positive. The negative Feulgen reaction would appear

to eliminate the possibility that deoxyribonucleic acid was present in detectable amounts. The negative periodic acid-Schiff reaction indicated the absence of complex carbohydrates. Moreover, no fat, lead, or iron could be demonstrated. Thus, by exclusion, it seems likely that these bodies are of protein nature.

The resemblance of the Lewy body to that seen in Pick's disease is quite striking. Greenfield and Bosanquet found that the only demontrable difference was the absence of a central core, a somewhat tenuous distinction which is not further clarified by more recent studies relying almost exclusively on silver impregnations.[22] The differentiation from the bodies observed in myoclonus epilepsy rests primarily on their metachromatic properties in the latter condition, first demonstrated by Lafora and Glueck.[23] The appearance of the GIB is somewhat reminiscent of the Negri body, but it does not exhibit the basophilic particles or the Feulgen-positive internal structure demonstrated by Moulton.[24] Other cytoplasmic inclusions, such as those of cytomegalic inclusion disease and the Guarnieri body of smallpox, are histochemically and morphologically quite distinct.[25,26]

The observations of the neuron which contains the Lewy body contribute to an understanding of this structure. In no case was a GIB found in an otherwise normal neuron. Evidence that the neuron had undergone some form of degeneration was indicated by eccentricity of the nucleus, less commonly encountered nuclear abnormalities, margination and loss of Nissl substance, apparent partial loss of pigment and characteristic arrangement of its remnants, and the occurrence of cytoplasmic vacuolation. In instances in which multiple GIB occurred within the same cell, it was not unusual for some of the bodies to exhibit variations suggestive of a progressive change. These observations, considered in conjunction with the data related to incidence and the histochemical features, suggest that the bodies represent a nonspecific form of degenerative alteration.

A final consideration bearing on this point is the preferential occurrence of the bodies in pigmented neurons. The appearance of pigmentation in the substantia nigra is phylogenically[27] as well as ontogenically[28] a relatively late event. While there is no available information as to the function of the pigment in these cells, it is certain that at one time or another in the early portion of the life span of the neuron, this substance must be formed. It is uncertain whether or not the process of formation is continuous, but it is not uncommon to observe in otherwise intact neurons in the substantia nigra of normal individuals, collections of granules of the same size and shape as the "neuromelanin" granules which are unpigmented and eosinophilic. They are similar if

not identical to those described many years ago by Marinesco[29] as an early manifestation of the process of pigmentation. In any case the pigmented ganglion cells of the substantia nigra must be considered either to be experiencing or to have experienced an additional metabolic demand not made upon other neurons of the central nervous system with the exception of those of the locus caeruleus and the dorsal vagal nucleus. These regions, perhaps not coincidentally, are the next most frequent sites of occurrence of the Lewy body.

Summary

Brain tissue from 53 patients suffering from parkinsonian states and 206 patients without parkinsonism, serving as controls, was systematically examined in relation to the occurrence of ganglionic intracytoplasmic bodies. These distinctive structures were found in the substantia nigra of 75 per cent of all cases with parkinsonian states (44 per cent of the cases with the postencephalitic variety and 86 per cent of those with the idiopathic variety), and in 4.9 per cent of the control series. Histochemical, morphologic and statistical data are presented, indicating the probability that these bodies represent a form of nonspecific neurocellular degeneration, possibly of abiotrophic nature.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

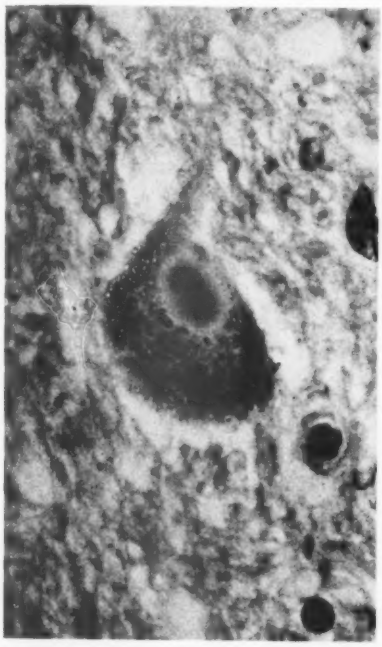
Except where indicated, illustrations were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Ganglion cell containing a single typical, well defined Lewy body. The nucleus is at the lower pole of the cell, out of the plane of focus. Postencephalitic parkinsonism. × 980.

FIG. 2. Neuron containing a larger though less dense Lewy body. The halo is less well defined. Postencephalitic parkinsonism. Substantia nigra. × 1200.

FIG. 3. Two neurons, both showing multiple ganglionic intracytoplasmic bodies, which vary in their densities, and the degree of definition of surrounding halos. Note the peripheral rim of remaining Nissl substance and the faintly granular but otherwise featureless appearance of the cytoplasm. Paralysis agitans. Substantia nigra. × 980.

FIG. 4. Two neurons in the locus caeruleus showing Lewy bodies with varying degrees of differentiation. Paralysis agitans. × 980.



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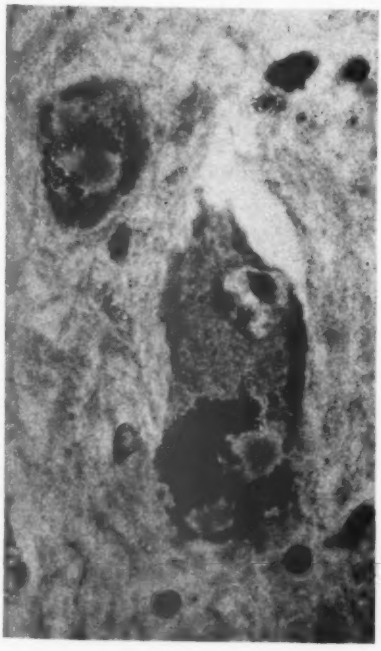

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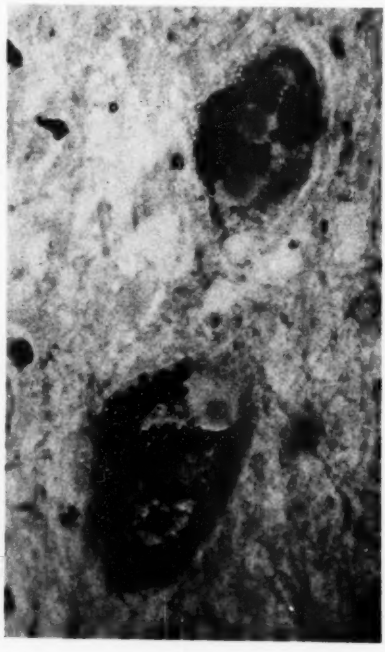

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FIG. 5. Neurons undergoing degenerative change containing 3 distinct GIB, of varying degrees of "maturation." The largest body has an unusually wide halo and shows a somewhat variable density. Two glial cells are in the immediate vicinity. Paralysis agitans. Substantia nigra. × 1200.

FIG. 6. Ganglion cell with a large cytoplasmic vacuole and 3 dense Lewy bodies. Note absence of "neuromelanin" pigment. Paralysis agitans. Pons. × 1200.

FIG. 7. Neuron, from dorsal vagal nucleus, with markedly eccentric ellipsoidal nucleus (N). The large GIB (edges indicated by arrows) shows a deeply chromatropophilic (red) central density. The cytoplasmic vacuolation encroaches upon the halo. Note the granularity of the cytoplasm. Paralysis agitans. Roque's chromotrope 2R-aniline blue stain. × 1200.

FIG. 8. A binucleate neuron (N) with GIB showing "frayed" irregular edges (arrows) and cytoplasmic vacuolation encroaching upon the halo. Paralysis agitans. Substantia nigra. Chromotrope 2R-aniline blue. × 1200.

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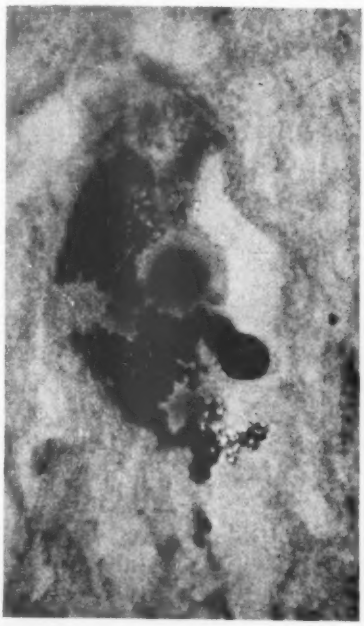

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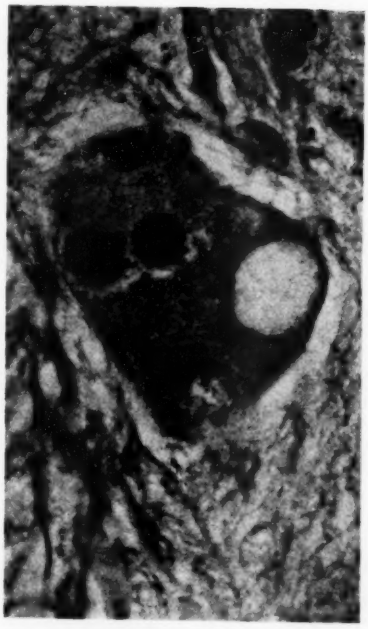

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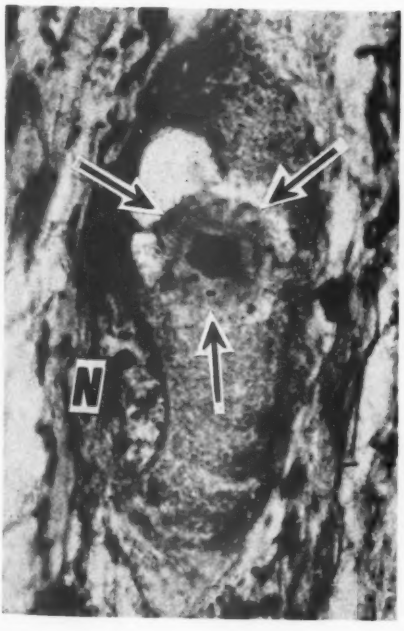

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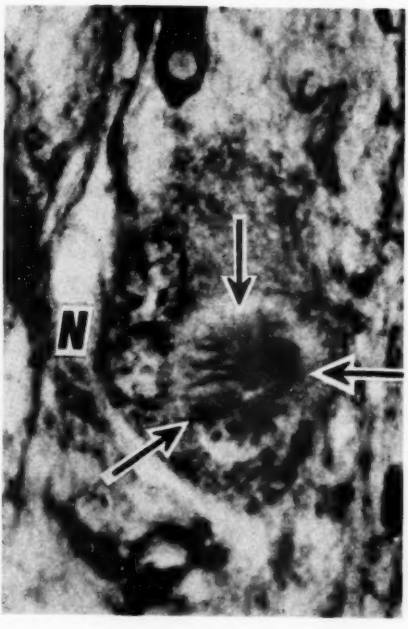

THE FINE STRUCTURE OF CEREBRAL FLUID ACCUMULATION

I. Swelling Secondary to Cold Injury *

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Cerebral fluid accumulation, both as to its structure and functional aspects, presents one of the many intriguing and elusive problems of neuropathology. Spatz[1] originally differentiated cerebral swelling from cerebral edema on the basis of the macroscopic appearance. Those specimens which were enlarged, rather firm and dry were considered to demonstrate cerebral swelling. Cerebral edema was, by definition, characterized by enlargement, softening and visible wetness. Cerebral swelling is commonly noted adjacent to intracranial neoplasms, whereas examples of edematous brains may be seen in uremia, alcoholism, and some instances of congestive heart failure. Furthermore, it was considered that cerebral swelling represented an excessive amount of intracellular fluid, while the abnormal accumulation was extracellular in the case of cerebral edema.

These apparent differences could not be rigorously verified by light microscopy, and confirmation was unobtainable even with modern histochemical techniques and biochemical analyses.[2] The prevalent concept, at least among Anglo-American investigators,[3-6] is that swelling and edema are fundamentally the same process, differing only in degree. According to this theory, a mildly excessive amount of fluid may be maintained within the cells. Greater fluid accumulation, however, results in enlargement of the extracellular space and is manifested by cerebral edema as defined and described by Spatz. It is generally agreed that the white matter is the major site of involvement in either condition. On the other hand, the problems of intracellular versus extracellular fluid accumulation, and the identification of the cells involved in these reactions remain as controversial today as they were 30 years ago.

Recent investigations of the central nervous system by means of electron microscopy have considerably extended knowledge of its basic anatomic structure. One of the most surprising observations[7-9] was that the cellular elements of the brain apparently fitted together so

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closely that the extracellular space amounted to no more than 1 or 2 per cent of the whole. The accumulation of fluid in the brain under experimental conditions should, therefore, visibly alter this compact arrangement.

Klatzo, Piraux and Laskowski[10] recently described localized cerebral edema secondary to cold injury. The application of electron microscopy to the investigation of such a lesion should reveal the location of the fluid and also contribute to a better understanding of fluid transfer in the brain.

Material and Methods

In a preliminary study 12 four-month-old albino mice were employed, utilizing a modification of Klatzo's technique for freezing a part of the brain. The animals were anesthetized with Nembutal,® and a 4 mm. hole was drilled through the parietal bone. Carbon dioxide ice was applied to the cerebrum for periods of 20 or 30 seconds. The animals were then sacrificed at short intervals thereafter. Histologic preparations revealed maximal alterations 24 to 72 hours after a 30-second application.

A more definitive study was performed using 36 four-month-old albino mice. Anesthesia and craniotomy were performed as before. Carbon dioxide ice was applied to the cerebrum for 30 seconds. In addition, 4 rats were treated in a similar manner. One tenth ml. of 1 per cent trypan blue solution was injected subcutaneously in 6 mice in order to demonstrate the passage of the vital dye across the blood-brain barrier into the injured cerebral tissue. Specimens were taken for electron microscopy at intervals of 24, 48 and 72 hours following the cold injury. This tissue was fixed for 2 hours in 2 per cent osmium tetroxide buffered with veronal acetate.[11] Following rapid dehydration, the tissue was embedded in methacrylate resin and sectioned with a Porter-Blum microtome (Servall). These sections were examined in RCA EMU 3-A and RCA EMU 3-D electron microscopes. Specimens for examination by light microscopy were taken from the same animals which were used for electron microscopy. Some animals were sacrificed at 5, 8, 12, 17 and 27-day intervals following the cold injury. The tissues for light microscopy were fixed in 10 per cent formalin and in Rossman's picric acid-alcohol fixative. Hematoxylin and eosin, periodic acid-Schiff (PAS), Nissl and Cajal stains were used.

Results

The fluid accumulation was manifested grossly by an enlargement of the affected cerebral hemisphere with an actual protrusion of the cerebrum through the craniotomy wound to produce a cerebral "fun-

gus." The lesion was stained vividly following injection of trypan blue, indicating a local breakdown of the blood-brain barrier. Microscopic examination of hematoxylin and eosin stained preparations revealed a loosening of the cerebral structure which was most prominent in the white matter (Figs. 1 and 2). Cellular swelling was not a prominent feature as seen with the light microscope in the mouse. Periodic acid-Schiff stained preparations (Fig. 3) showed granular PAS-positive substance which was proportional in amount to the severity of the edema. This substance was identified in perivascular spaces and within cell bodies. There was also considerable material dispersed in the remainder of the adjacent cortex and white matter. Here it could not be determined whether it was extracellular or intracellular. The maximum reaction occurred between 24 and 72 hours following injury. There was then gradual and progressive resolution of the fluid accumulation and healing of the superficial necrotic zone. At 27 days only a small glial scar remained.

Electron microscopic examinations of normal cerebral mouse tissue confirm the observations of previous investigators[7-9] with regard to the apparent absence of an extracellular space. The perivascular spaces seen in light microscopy are found to be largely artifacts. The clear zone which surrounds small vessels is composed of relatively clear processes which are closely applied to the outer surfaces of the blood vessel walls (Fig. 4). These processes are positively identified as cellular extensions since they contain mitochondria and endoplasmic reticulum. The white matter not in contiguity with blood vessels contains similar clear processes which are relatively inconspicuous (Fig. 5). In these locations glial cell bodies with watery cytoplasm similar to that of the processes are also present (Fig. 6). Because of the similarity in density and content, the clear processes are considered to be extensions of the clear cell bodies. These cells will be referred to as "clear cells" to distinguish them from neurons and microglia which have dense cytoplasm. This descriptive label also distinguishes this cell from the type of glial element which has dense cytoplasm and small processes. The term also avoids the controversy[7-9,12] concerning the identification of these cells. The cell processes, cell bodies, myelinated and non-myelinated nerve fibers and blood vessels are very closely approximated with the result that only a minute (about 10 mμ) intercellular space is visible.

Examination of the cold injury lesion by means of the electron microscope also revealed the predominant alteration to be within the white matter and neuropil. The changes observed in the mouse and in the rat were similar. However, in the latter the maximum alteration was not

attained until 48 hours after the injury. In both species the most striking feature was enlargement of the cytoplasmic volume of the clear glial cells. The clear zones surrounding blood vessels and the enlarged processes at a distance from vessels were identified as cellular extensions, mainly by virtue of their content (Figs. 7 to 9). Mitochondria and endoplasmic reticulum were consistently present in these areas. Although the entire cell appeared to enlarge virtually simultaneously, swelling was somewhat more prominent in the regions adjacent to blood vessels. All processes appeared to have intact limiting cell membranes. Fluid accumulation between cells was never seen. Significant enlargement or alteration of cells other than the clear type was not observed.

Discussion

A cerebral extracellular space has been believed to exist because of certain morphologic and some chemical evidence. Perivascular and perineuronal spaces were visible when fixed tissue was embedded in paraffin or celloidin and was examined by the light microscope. Improved fixation and the superior resolving power of the electron microscope served to demonstrate that these zones were filled by glial cell processes. A cerebral ground substance was postulated by many early workers and was demonstrated most recently by Hess[13] in periodic acid-Schiff stained preparations. This ground substance, too, was now seen to be fully occupied by cellular elements. Lumsden[14] demonstrated that glia may contain PAS-positive material. Chloride, inulin and thiocyanate, useful in other organs for measuring the extracellular compartment, have been found[15] to diffuse only very slowly into the brain. Such determinations also suggested that the cerebral intercellular compartment was of a different nature from that of other viscera.

A small intercellular space certainly does exist in both normal and experimentally swollen brains. As seen with the electron microscope, adjacent cell membranes are separated by a gap of 10 to 20 mμ. This dimension remains remarkably constant despite the irregular contours of the cellular elements. It is possible that this space, even though minute, may be physiologically significant in fluid transport. But this hypothesis becomes less plausible in view of the apparent failure of the space to enlarge in the presence of an increased quantity of fluid in the brain, as in the experimental conditions described.

As previously mentioned, neuropathologists have divided the accumulation of fluid within the brain into cerebral swelling and cerebral edema largely on the basis of gross appearances. The fluid accumulation secondary to cold injury appears to be intracellular and would thus conform to the traditional concept of cerebral swelling. The

traditional concept also maintains that in cerebral edema the accumulated fluid is extracellular. Can such a viewpoint be tenable, considering the mounting evidence that there is no significant extracellular space under normal circumstances or in cerebral swelling?

In other organs, whenever there is excess fluid, it is found most prominently in the interstitial tissue, especially around the vessels, which are its source. In the brain, however, this fluid is found in glial processes, and somewhat more prominently in those cellular extensions which surround the blood vessels. It is well to note that the capillary basement membrane is closely invested by glial processes, both of the dense and clear types. When the brain is swollen, the clear processes enlarge. This leads to the suggestion that the clear cells have a vital role in fluid transport, comparable to the function of the extracellular space in other tissues. These cells may then also be involved in electrolyte transfer, nutrient supply and waste removal.

The identity of the glia with the clear cytoplasm is uncertain in terms of classical neurohistology. Farquhar,[7] Schultz,[8], DeRobertis,[16] and their co-workers consider them to be astrocytes. On the other hand, Luse[12] and Palay[17] maintain that they are oligodendroglia. Whatever their name, it seems apparent that the clear glial cells have a vital role in fluid transport and that their plasma membrane is an important component of the blood-brain barrier.

Conclusions

1. The cerebral intercellular space is normally very small (10 to 20 mμ wide).

2. Application of carbon dioxide ice to the exposed brain of the mouse results in localized cerebral swelling, manifested by enlargement of the cytoplasmic volume of that type of glia which has clear cytoplasm.

3. The cerebral swelling does not extend to or involve the intercellular space which remains very narrow.

4. It is suggested that the clear glia provide an intracellular transport system for nutrients and electrolytes and that this system functions in the cerebrum as the extracellular space does in other organs.

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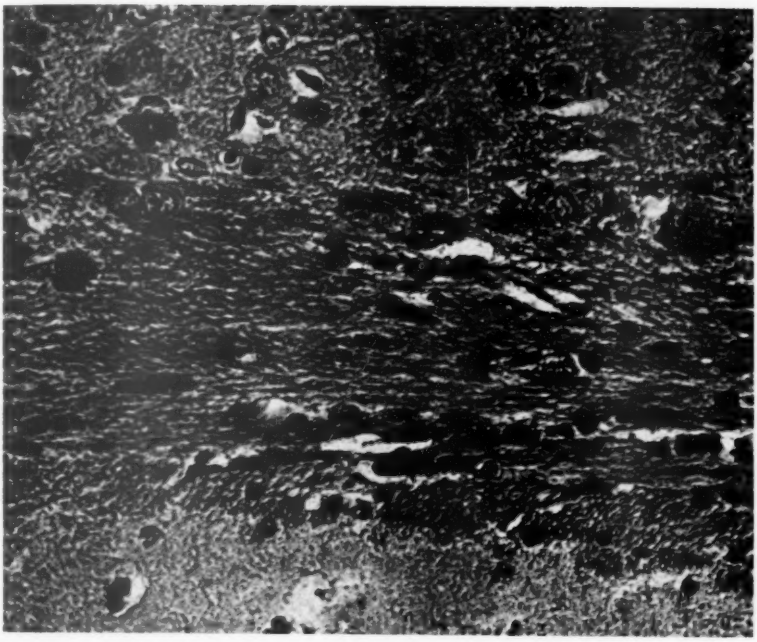
Mrs. Beverly Padwo and Mr. Martin Besen provided technical assistance, and Mr. Antol Herskovitz prepared the conventional photomicrographs.

LEGENDS FOR FIGURES

FIG. 1. Mouse cerebrum. Normal corpus callosum. Hematoxylin and eosin stain. × 450.

FIG. 2. Mouse cerebrum. Swollen corpus callosum underlying the CO_2 lesion. Hematoxylin and eosin stain. × 450.



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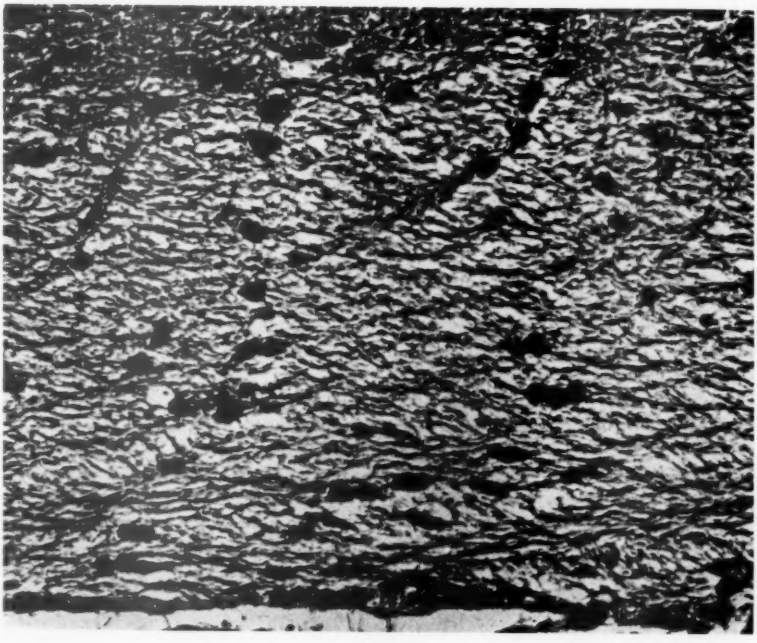

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FIG. 3. Mouse cerebrum. Superficial necrotic zone with adjacent swelling. Periodic acid-Schiff stain. × 150.

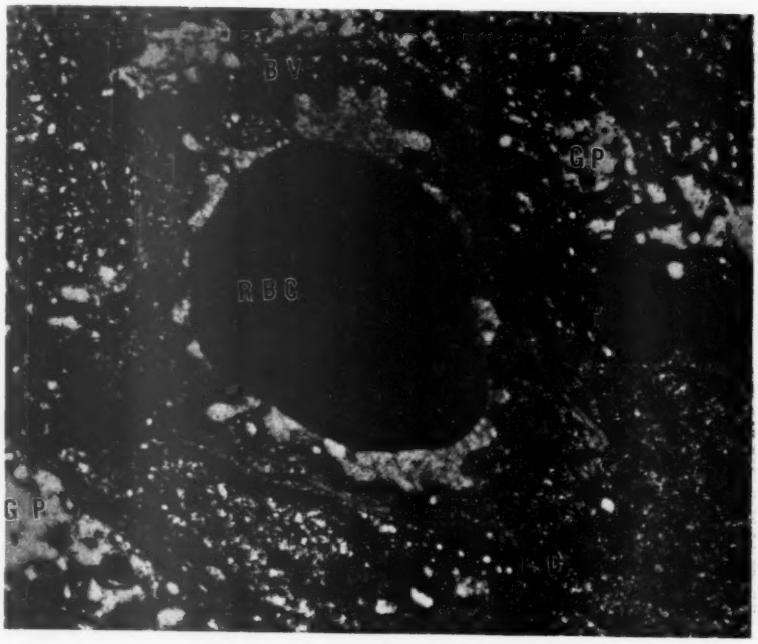
Figures 4 to 9 are electron micrographs.

Key:

BV = blood vessel	CGN = clear glial cell nucleus
EC = endothelial cell	CGC = clear glial cell cytoplasm
RBC = red blood cell	ER = endoplasmic reticulum
GP = clear glial processes	M = mitochondrion
MNF = myelinated nerve fiber	WBC = white blood cell

FIG. 4. Mouse cerebrum. Normal blood vessel. × 10,600.

FIG. 5. Normal mouse cerebrum. × 10,600.



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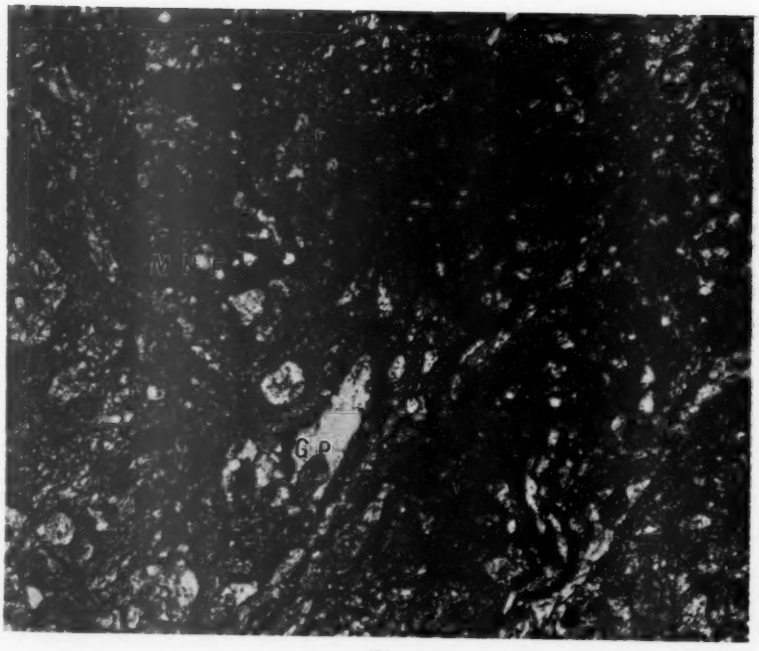

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FIG. 6. Mouse cerebrum. Normal clear glial cell body. × 10,300.

FIG. 7. Swollen mouse cerebrum. × 10,600.

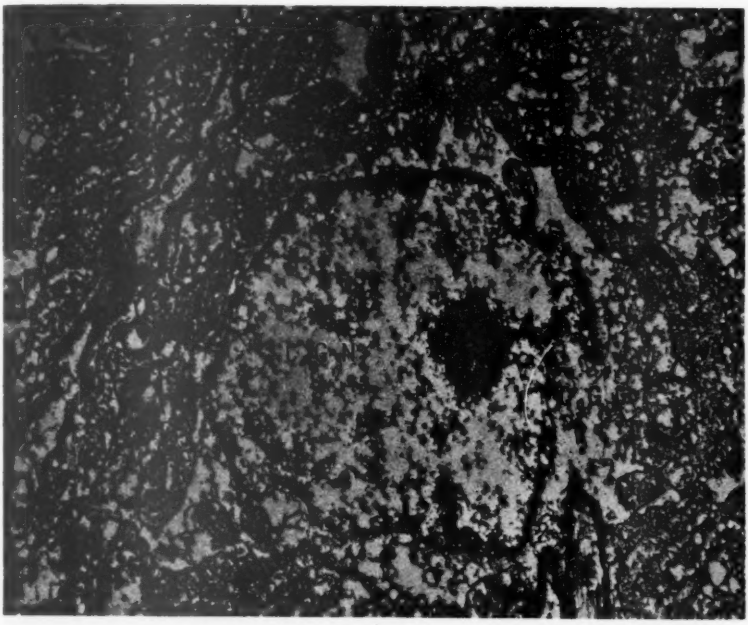

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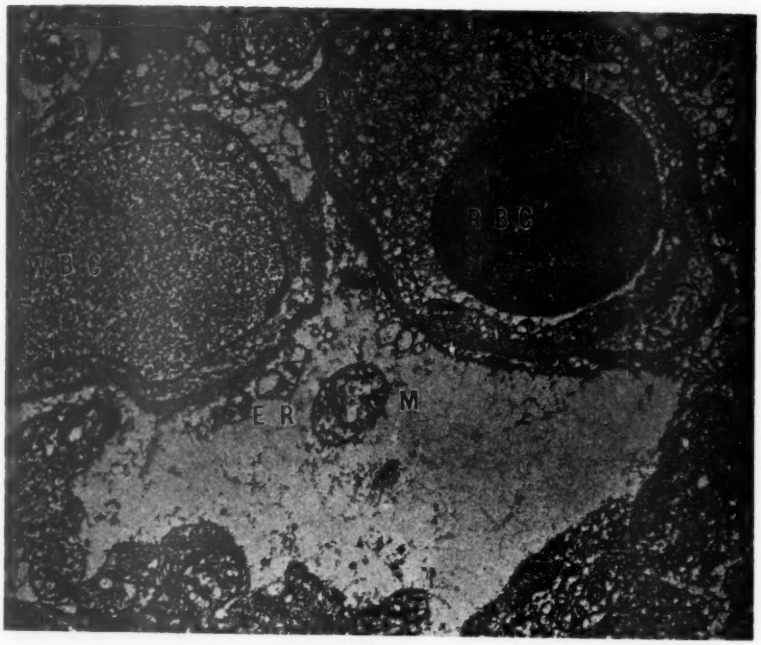
FIG. 8. Swollen mouse cerebrum with dilated perivascular glial processes. × 10,600.

FIG. 9. Swollen mouse cerebrum. × 10,600.



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9

STUDIES ON EXPERIMENTAL SHOCK

Effects of Vasopressor Amines and Phenothiazine Derivatives*

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In a previous study[1] it was found that a 20-minute period of rotation in a modified Noble-Collip drum (480 to 500 revolutions) consistently produced a state of prostration and exhaustion in rabbits, and that 10 to 20 per cent of the animals died within 12 hours following the procedure. The salient morphologic features observed in the animals were cardiac valvular hemorrhages, myocardial necrosis, and hemorrhagic and ischemic lesions of the small intestine.

When rotation was performed in conjunction with an intravenous injection of gram-negative endotoxin, the lethality was increased markedly, and the cardiovascular lesions were associated with deposits of hyaline fibrinoid material. In addition, approximately 45 per cent of the animals developed extensive areas of hepatic necrosis.[1] Similarly, it has been shown that a period of acute rotational shock may alter the response of rabbits to infection. The lethal effects, as well as the incidence and severity of lesions, are increased if the procedure is performed in association with either an intravenous or a pharyngeal injection of beta hemolytic streptococci.[2] Furthermore, recent studies indicate that the acute shock technique renders rabbits susceptible to infection with micro-organisms which are nonpathogenic for normal animals.[3]

In an effort to obtain further information concerning the mechanisms involved in the production of rotational shock, and to study in greater detail the progression of associated lesions, it seemed desirable to effect some method by which the stress period could be lengthened without a concomitant increase in fatality. The clinical use of vasopressor amines in shock suggested their use in this experimental procedure. Zweifach and associates[4,5] reported that chlorpromazine, a phenothiazine derivative, effectively reduced the lethality of both traumatic and hemorrhagic shock in rats, and this report led us to investigate the effects of certain of these drugs in rotational shock in rabbits.

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Material and Methods

Hybrid albino rabbits of both sexes, weighing approximately 1 kg., were used in the experiments. They were fed Purina rabbit pellets and had free access to water. Acute rotational shock was produced by a modified Noble-Collip drum, as described previously.[1] The animals were subjected to rotation in groups of 4, of which 1 or 2 of each group served as controls. It was found that a 30-minute period of rotation (800 to 900 revolutions) invariably was fatal for untreated animals, and this period was used throughout the experiments.

A total of 584 rabbits was used in the investigation. Of these, 180 were used in the experiments involving sympathomimetic amines, and 404 were used in studies on the phenothiazine derivatives.

The following sympathomimetic amines were used: levarterenol (Levophed®), epinephrine (Adrenalin®), phenylephrine (Neo-Synephrine®), metaraminol bitartrate (Aramine), and mephentermine (Wyamine®). Levarterenol and epinephrine were given in a dosage of 20 μg. per kg. of body weight, phenylephrine and metaraminol in a dosage of 200 μg. per kg., and mephentermine in a dosage of 10 mg. per kg.

The phenothiazine derivatives were given singly or in combination in a dosage of 5 mg. per kg. each. These included promazine (Sparine®), chlorpromazine (Thorazine®), promethazine (Phenergan®), mepazine (Pacatal®), prochlorperazine (Compazine®), and WY 1359, generously supplied by Wyeth Laboratories.

All injections were given into the marginal ear vein, in a volume of 1 ml. Control animals were given an injection of 1 ml. of sterile isotonic saline solution. The drugs were administered one hour prior to drum rotation (phenothiazines only), immediately prior to rotation, or during the 30-minute period of rotation. In the last instance, the animals were rotated for a given period of time, removed from the drum and treated, then replaced in the drum for the remainder of the 30-minute period. The numbers of animals used in the various experiments are summarized in the tables.

The animals which did not die were sacrificed 24 hours to 10 days following the shock procedure. Necropsy examinations were performed, and the tissues were fixed in 10 per cent neutral formalin. Histologic sections were stained with hematoxylin and eosin.

Results

Effects on Lethality

The results obtained by administration of vasopressor amines in rotational shock are summarized in Tables I and II. As shown in

Table I, of 40 animals given one of the sympathomimetic amines simultaneously with drum shock, 32 (80 per cent) died during the period of rotation, and only 5 (13 per cent) survived more than 24 hours following the procedure.

TABLE I

Influence on Survival Time of Rabbits Given Vasopressor Amines Simultaneously with Drum Rotation

Drug	Dosage	No. of animals	No. dying				No. living more than 24 hr.
			During rotation	Less than 1 hr.	Less than 4 hr.	Less than 8 hr.	
Levarterenol	20 μg.	8	6	0	0	0	2 (25%)
Phenylephrine	200 μg.	8	5	1	0	0	2 (25%)
Epinephrine	20 μg.	8	6	0	1	0	1 (13%)
Metaraminol	200 μg.	8	7	0	1	0	0 (0%)
Mephentermine	10 mg.	8	8	0	0	0	0 (0%)
Saline (controls)	1 cc.	14	14	0	0	0	0 (0%)

TABLE II

Influence on Survival Time of Rabbits Given Vasopressor Amines at Varied Times During Interrupted Rotational Shock

Duration of rotation		Drug and dosage*	No. of animals	No. dying				No. living more than 24 hr.
Before treatment	After treatment			During rotation	Less than 1 hr.	Less than 4 hr.	Less than 8 hr.	
5 min.	25 min.	Epinephrine	8	7	1	0	0	0
		Levarterenol	8	7	0	0	0	1
		Metaraminol	7	6	0	0	0	1
		Mephentermine	7	6	0	0	1	0
		Phenylephrine	6	6	0	0	0	0
		Saline, 1 ml.	6	6	0	0	0	0
10 min.	20 min.	Epinephrine	8	4	1	2	0	1
		Levarterenol	8	6	1	0	0	1
		Metaraminol	7	5	1	0	0	1
		Mephentermine	7	6	0	1	0	0
		Phenylephrine	6	6	0	0	0	0
		Saline, 1 ml.	6	6	0	0	0	0
15 min.	15 min.	Epinephrine	8	6	1	1	0	0
		Levarterenol	8	7	1	0	0	0
		Metaraminol	7	6	0	1	0	0
		Mephentermine	7	7	0	0	0	0
		Phenylephrine	6	5	1	0	0	0
		Saline, 1 ml.	6	5	1	0	0	0

* Epinephrine or levarterenol, 20 μg. per kg. of body weight; metaraminol or phenylephrine, 200 μg. per kg.; mephentermine, 10 mg. per kg.

Similarly, as shown in Table II, of 108 animals given a vasopressor amine in conjunction with interrupted rotation, 90 (83 per cent) died during the continuance of rotation after the drug was given, and only 5 (4 per cent) survived more than 24 hours. Although there is little

TABLE III

Influence on Survival Time of Rabbits Given Various Phenothiazine Derivatives One Hour Prior to Drum Rotation

Drug*	No. of animals	No. dying				No. living more than 24 hr.
		During rotation	Less than 1 hr.	Less than 4 hr.	Less than 8 hr.	
Promazine (Sparine®)	10	2	0	2	1	5 (50%)
Chlorpromazine (Thorazine®)	10	3	1	0	1	5 (50%)
Mepazine (Pacatal®)	6	5	1	0	0	0 (0%)
Promethazine (Phenergan®)	6	6	0	0	0	0 (0%)
WY 1359	6	6	0	0	0	0 (0%)
Saline, 1 cc. (controls)	14	14	0	0	0	0 (0%)

* Each drug was given in a dosage of 5 mg. per kg. of body weight.

TABLE IV

Influence on Survival Time of Rabbits Given Combinations of Various Phenothiazine Derivatives One Hour Prior to Drum Rotation

Drug*	No. of animals	No. dying				No. living more than 24 hr.
		During rotation	Less than 1 hr.	Less than 4 hr.	Less than 8 hr.	
Promethazine + promazine	14	0	0	2	0	12 (86%)
WY 1359 + promazine	12	1	1	0	0	10 (83%)
Promethazine + chlorpromazine	12	6	1	0	0	5 (42%)
Promethazine + mepazine	6	3	1	1	0	1 (16%)
Saline, 1 cc. (controls)	18	18	0	0	0	0 (0%)

* Each drug was given in a dosage of 5 mg. per kg. of body weight.

difference among the various drugs given with respect to their inability to alter the lethal effects of the procedure, it is of interest that only 1 of 39 animals given either mephentermine or phenylephrine during interrupted rotation survived longer than 8 hours after the procedure.

Tables III to VII summarize the results obtained by administration of the phenothiazine derivatives in conjunction with drum rotation. Only two of the drugs, promazine and chlorpromazine, were effective in reducing the mortality when given one hour prior to drum rotation. With each of these, a survival rate of 50 per cent was obtained (Table

III). As shown in Table IV, however, survival rates of 83 to 86 per cent were obtained when promazine was administered in combination with either promethazine or WY 1359 one hour prior to rotation. The combination of promethazine with chlorpromazine, on the other hand,

TABLE V

Influence on Survival Time of Rabbits Given Various Phenothiazine Derivatives Simultaneously with Drum Rotation

Drug*	No. of animals	No. dying				No. living more than 24 hr.
		During rotation	Less than 1 hr.	Less than 4 hr.	Less than 8 hr.	
WY 1359	14	0	1	0	1	12 (86%)
Chlorpromazine (Thorazine®)	14	3	0	1	2	8 (57%)
Promazine (Sparine®)	14	1	0	3	3	7 (50%)
Prochlorperazine (Compazine®)	6	4	0	0	1	1 (16%)
Mepazine (Pacatal®)	6	4	2	0	0	0 (0%)
Promethazine (Phenergan®)	6	6	0	0	0	0 (0%)
Saline, 1 cc. (controls)	22	22	0	0	0	0 (0%)

* Each drug was given in a dosage of 5 mg. per kg. of body weight.

TABLE VI

Influence on Survival Time of Rabbits Given Combinations of Various Phenothiazine Derivatives Simultaneously with Drum Rotation

Drug*	No. of animals	No. dying				No. living more than 24 hr.
		During rotation	Less than 1 hr.	Less than 4 hr.	Less than 8 hr.	
WY 1359 + promazine	10	0	1	0	1	8 (80%)
Promethazine + promazine	10	3	1	0	0	6 (60%)
Promethazine + chlorpromazine	10	5	0	2	0	3 (30%)
Promethazine + mepazine	6	5	0	0	0	1 (16%)
Promethazine + prochlorperazine	6	5	0	0	1	0 (0%)
Promethazine + WY 1359	6	6	0	0	0	0 (0%)
Saline, 1 cc. (controls)	18	18	0	0	0	0 (0%)

* Each drug was given in a dosage of 5 mg. per kg. of body weight.

did not appreciably alter the results compared to those obtained with chlorpromazine alone. This is of some interest in view of the observation that both promethazine and WY 1359 are completely ineffective in preventing death when given alone one hour prior to drum rotation (Table III).

When single drugs were administered immediately before the onset of drum rotation, 86 per cent of the animals given WY 1359 survived, as compared to a survival rate of 57 per cent with chlorpromazine, and 50 per cent with promazine. The results obtained with mepazine or promethazine were comparable to those obtained when these drugs were given one hour prior to rotation, in that none of the animals survived more than one hour following rotation.

The survival rate obtained by administration of drug combinations simultaneously with drum rotation (Table VI) was not appreciably

TABLE VII

Influence on Survival Time of Rabbits Given Phenothiazine Derivatives at Varied Times During Interrupted Rotational Shock

Interval	Drug*	No. of animals	No. dying				No. living more than 24 hr.
			During rotation	Less than 1 hr.	Less than 4 hr.	Less than 8 hr.	
After 5 min. rotation	WY 1359	18	4	2	2	0	10 (55%)
	Chlorpromazine	18	10	0	0	0	8 (44%)
	Promazine + promethazine	6	5	1	0	0	0 (0%)
	Saline (controls)	14	13	1	0	0	0 (0%)
After 10 min. rotation	WY 1359	14	5	1	0	0	8 (57%)
	Chlorpromazine	14	7	0	0	0	7 (50%)
	Promazine + promethazine	6	6	0	0	0	0 (0%)
	Saline (controls)	12	12	0	0	0	0 (0%)
After 15 min. rotation	WY 1359	14	10	1	0	0	3 (21%)
	Chlorpromazine	14	8	1	0	0	5 (36%)
	Saline (controls)	12	11	1	0	0	0 (0%)

* Each drug was given in a dosage of 5 mg. per kg. of body weight.

greater than that observed when single drugs were given at this interval (Table V). Indeed, the combination of promethazine and chlorpromazine was actually less effective in this regard, and the combination of WY 1359 with promethazine was completely ineffective in preventing death.

Drugs which had provided the best protection when given simultaneously with rotation were selected for use in animals in which the period of rotation was interrupted for treatment and then continued so that a total time of 30 minutes elapsed in the drum. These studies, summarized in Table VII, show that the combination of promazine and promethazine was completely ineffective in preventing death, once the shock state had been induced. On the other hand, survival rates as

high as 57 per cent were obtained with WY 1359, and 50 per cent with chlorpromazine. The number of survivors, however, dropped sharply when the drug was withheld until 15 minutes after rotation had been in progress.

Morphologic Alterations

Structural alterations in the lungs, heart, small intestine, and gallbladder were observed in the animals from both groups. In addition, in those given one of the vasopressor amines, liver lesions were noted. The gross lesions consisted of pulmonary hemorrhage and edema, atelectasis, and hemorrhages in the heart and small intestine. The gallbladder displayed foci of necrosis to which, in many instances, the omentum was attached by fibrinous adhesions. In several instances perforation had occurred, with leakage of bile into the surrounding omental fat. In the animals given one of the vasopressor amines, the liver showed pale to yellow, opaque, focal to confluent areas of necrosis scattered throughout the parenchyma.

Microscopic examination of the lungs showed extensive congestion, hemorrhage and edema in animals which died during rotation or shortly thereafter. In those that died 2 to 8 hours after rotation, varying degrees of atelectasis were observed. In several instances the areas of alveolar collapse were associated with widely dilated respiratory bronchioles and alveolar ducts. Except for the absence of hyaline deposit, the type of unequal expansion closely resembled that of hyaline membrane disease of the newborn (Fig. 1). No significant pulmonary lesions were observed in animals killed 24 hours or more after rotation.

Cardiac alterations observed in the animals which died at short intervals following rotation consisted of myocardial and valvular edema and hemorrhage (Fig. 2). In those sacrificed 24 hours or more following rotation, there was extensive muscle necrosis and inflammatory reaction, associated with varied degrees of resolution of the necrotic tissues (Fig. 3). In occasional animals, particularly those sacrificed 6 to 10 days after rotation, the intramural coronary arteries showed a marked degree of endothelial proliferation, with accompanying perivascular collections of mononuclear cells (Fig. 4). These lesions in the myocardium and valves were associated occasionally with deposits of hyaline fibrinoid material.

Microscopically, the small intestine exhibited areas of intense mucosal congestion and intraluminal hemorrhage. In animals that died between 4 and 24 hours following rotation, these lesions were associated with areas of intestinal infarction (Fig. 5). No significant lesions were observed in the intestine in the animals which were sacrificed more than 24 hours following rotation.

The gallbladder contained areas of complete, ischemic necrosis. These usually were limited to the outer portion of the organ, distal to the liver, but occasionally involved the complete circumference (Fig. 6). The lesion was recognizable in animals which died within 2 to 3 hours following rotation, and was accompanied by a crescent-shaped zone of hepatic necrosis adjacent to the gallbladder (Fig. 7). In animals sacrificed 24 hours after rotation, a varying degree of cellular reaction about the necrotic gallbladder wall was noted, and the adjacent vessels showed extensive subendothelial accumulations of fibrinoid material. The cellular reaction in the animals given vasopressor substances was quite extensive (Fig. 8), but was exceedingly sparse in those given a phenothiazine derivative. Serial investigation of the gallbladder lesion, in animals sacrificed over a period of 2 to 10 days after rotation, in many instances revealed a complete restoration of epithelium over the necrotic zone and proliferation of fibrous tissue within and around the gallbladder wall. In other instances the areas of necrosis extended into the surrounding omentum; in this case there was an exceedingly heavy accumulation of inflammatory cells (Figs. 9 to 12).

Aside from the previously mentioned hepatic necrosis adjacent to the gallbladder, the liver in animals given one of the phenothiazine derivatives appeared normal. In those given one of the sympathomimetic amines, focal or confluent zones of ischemic necrosis were observed. These often involved several lobules. There was little inflammatory reaction, but an extensive degree of calcification of the necrotic liver cells was noted in several instances.

The spleen, kidneys, pancreas, adrenal gland, skeletal muscle, and brain showed no constant microscopic lesions, although scattered cerebral petechiae were noted in several of the animals.

Discussion

Previous studies have shown that a majority of untreated rabbits tolerate without incident a 20-minute period of drum rotation. This procedure, however, becomes highly lethal when performed in conjunction with the administration of gram-negative endotoxin, or if the animals are infected with certain micro-organisms.[1,2] This increased fatality led us to seek some means of prolonging the survival time of rotated animals in order that any late manifestations of the procedure could be investigated. Consequently, because of their use in clinical shock, vasopressor amines were administered in a series of experiments to rabbits subjected to a lethal period of drum rotation.

The results obtained by the use of these substances were singularly disappointing. Only 13 per cent of the animals survived when a

sympathomimetic drug was administered immediately prior to rotation, and only 4 per cent survived when the drugs were given after the shock state had been induced. It is possible that different dosages or different methods of administration of the drugs might have modified these poor results. Under the conditions of the experiment, however, it appeared impractical to give more than a single intravenous injection, and the dosages used corresponded roughly with those used in other types of shock. The possibility exists, however, that rotational shock differs from other types of shock in which the vasopressor amines are commonly effective.

Nevertheless, it seemed desirable to search for a more effective method of preventing death under these experimental conditions. Previous reports by Zweifach and associates[4,5] concerning the use of chlorpromazine in traumatic or drum shock in rats suggested that the phenothiazine derivatives might be worthy of investigation in rabbits. In contrast to the results obtained with the sympathomimetic amines, it was found that survival rates as high as 86 per cent could be obtained if certain of the phenothiazine derivatives were administered one hour before, or immedately prior to, rotation in the drum. It was found, further, that certain of the drugs were effective even after the shock state had been induced, and that from 50 to 57 per cent of the animals survived an otherwise fatal period of rotation if treated not later than 10 minutes after rotation was begun.

Although these drugs resemble each other in their basic chemical structure, the results of the present study indicate marked differences in their ability to prolong the survival time of the animals subjected to drum shock. It was found, for example, that neither mepazine nor promethazine was effective in preventing death of the animals. Chlorpromazine and promazine, on the other hand, given one hour before or immediately before rotation, enabled 50 per cent or more of the animals to withstand the lethal procedure. Other studies indicated that these drugs also prevented death if given to rabbits as long as 4 hours prior to drum rotation.[6] These data emphasize the rapid effectiveness and long duration of action of both of the drugs. In contrast to these results, WY 1359, which failed to prevent death when given one hour prior to drum rotation, was highly effective when given simultaneously with the commencement of rotation, suggesting that this drug has a rapid onset of action but that its duration of action is short.

In an analagous fashion, combinations of different phenothiazines varied in their effects on the survival time of the animals. Promethazine and promazine proved highly effective when given in combination one hour prior to the rotational procedure, and 86 per cent of the animals

survived. On the contrary, when the combination of promethazine-chlorpromazine was given, the number of survivors decreased, and when promethazine was given in combination with WY 1359, none of the animals survived the period of rotation.

It is also of interest that the mixture of promethazine-promazine, highly effective in preventing death when given prior to drum rotation, failed to prevent death when administered to animals after the shock state had been induced. On the other hand, both chlorpromazine and WY 1359 were still effective in preventing death under these conditions.

The fundamental mechanisms by which the phenothiazine derivatives act to prevent the lethal effects of drum shock are not known. Several possibilities are suggested, however, by the results of these and other studies. The variations in survival rates obtained with the individual drugs of this group suggest that the side chain rather than the ring structure of the molecule may be the important factor, since each has a similar ring structure. In other studies, however, it has been shown that the administration of a sulfoxy phenothiazine derivative, containing a side chain identical with that of promazine, is completely without benefit in preventing death of animals subjected to drum shock.[6] Further, the fact that prevention of drum shock fatality is not a unique feature of phenothiazine derivatives is shown by the observation that a Rauwolfia derivative, reserpine (Serpasil®), is also effective in this regard if given one hour prior to drum rotation.[6] These results simply suggest that both portions of the molecule are necessary to produce a favorable result in experiments of this type.

The ability of the phenothiazine derivatives and the Rauwolfia compounds to produce a deep calming and sedative effect is well known, and the possibility that this action enables the animals to survive rotation must be considered. It should be emphasized, however, that although prochlorperazine (Compazine®) and mepazine (Pacatal®) are major tranquilizing agents, they fail to exert any beneficial effect when given in conjunction with drum shock. Similarly, the administration of analgesic agents such as meperidine (Demerol®) is without benefit. It seems therefore, that the tranquil state *per se* is not of primary importance in this regard.

The antihistaminic activity of the phenothiazine group of tranquilizers is well known, and this action should be considered in interpreting the present results. That this factor is of little or no importance is shown by the fact that promethazine, a phenothiazine derivative with marked antihistaminic activity, is nevertheless ineffective in preventing death of shocked animals when given alone. Similarly, the administration of diphenhydramine (Benadryl®) is not associated with

any protective action when given alone, and when given in combination with promazine, the number of survivors is less than that obtained by administration of promazine alone.[6]

In other studies it was observed that rabbits acclimated to a cold environment were able to withstand a far greater period of drum rotation than normal rabbits. It is known that chlorpromazine, and possibly other phenothiazine derivatives, reduce body temperature,[7] but it is doubtful that this action plays an important role in preventing drum shock death because of the rapidity with which the drugs act under these circumstances.

The rapidity of onset of action displayed by certain of the drugs used in the present study, and their effectiveness in preventing death even after shock had been induced, suggests a peripheral action as well as an effect on the central nervous system. Although the role of serotonin in the pathogenesis of shock is controversial, it is of interest that both reserpine and chlorpromazine block the peripheral effects of this indole amine.[8]

However, the administration of a diphenylmethane derivative, hydroxyzine (Vistaril[T.M.]), also known to block the peripheral effects of serotonin, did not protect animals from drum shock death when given alone or in combination with promethazine.[9] In this regard it would be of interest to investigate the effects on drum shock lethality of another diphenylmethane derivative, azacyclonol (Frenquel®), which has been shown also to block the actions of serotonin.[8]

A peripheral action of chlorpromazine in depressing both the sympathetic and parasympathetic nerve centers has also been established,[10] and it is possible that other phenothiazine derivatives, or combinations thereof, may have a similar action. It is significant in this regard that chlorpromazine prevents death in over 30 per cent of animals even after 15 minutes of rotational shock, whereas administration of sympathomimetic amines under similar circumstances is associated with a mortality of 100 per cent.

Another possibility to be considered with respect to the phenothiazine derivatives is their effect on capillary permeability. It has been demonstrated that chlorpromazine,[11] promethazine,[12,13] and WY 1359[14] prevent or delay leakage of protein through capillary walls. This action may play an important role in the prevention of death associated with rotational shock.

Although the definitive mechanisms through which the phenothiazines act to prevent drum shock death are still obscure, these observations and other available data suggest that a partial explanation of such action may be their depressant effects on the autonomic nervous sys-

tem, and their action in maintaining a normal or more normal state of capillary permeability. Whether these drugs will exert a beneficial effect in other types of shock, or in clinical conditions accompanied by shock, awaits further investigation.

Summary and Conclusions

Hybrid albino rabbits were subjected to a lethal period of rotation in a modified Noble-Collip drum, in conjunction with the administration of a vasopressor amine or certain phenothiazine derivatives.

Only 13 per cent of the animals survived when given a vasopressor simultaneously with the onset of rotation, and only 4 per cent survived when a vasopressor amine was given after the shock state had been induced. In contrast, survival rates of 83 to 86 per cent were obtained by administration of a combination of promethazine and promazine, or promazine and WY 1359, one hour prior to rotation. Fifty to 57 per cent of the animals survived when given promazine or chlorpromazine, and 86 per cent survived when WY 1359 was administered simultaneously with the start of drum rotation. Efficacy of the drugs in preventing death decreased when they were given after shock had been induced, although more than 50 per cent of the animals survived if given chlorpromazine or WY 1359 not longer than 10 minutes after shock was induced.

The lesions observed in both groups of animals included pulmonary hemorrhage and edema, atelectasis, cardiac muscle and valvular hemorrhages, and myocardial necrosis. In animals which died at short intervals following rotation, intestinal infarcts were encountered, and ischemic necrosis of the gallbladder occurred in a high percentage of the animals. In addition, in animals given a sympathomimetic amine, areas of hepatic necrosis were noted.

The significance of these results, and possible methods by which the phenothiazines act to prevent the lethality of rotational shock, are discussed. Available data suggest that important factors in this regard are their effects on the autonomic nervous system, and their actions on capillary permeability.

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The photographs were made by Mr. Henry Morris.

[*Illustrations follow*]

LEGENDS FOR FIGURES

All sections were stained with hematoxylin and eosin.

FIG. 1. Lung section from an animal dying 5 hours after drum rotation. The alveoli are collapsed, but the respiratory bronchioles and alveolar ducts are widely dilated. × 40.

FIG. 2. Mitral valve from an animal sacrificed 2 days after rotational shock. In addition to hemorrhage, accumulations of fibrinoid substance are also present in the valve. × 40.

FIG. 3. Section from the myocardium of an animal sacrificed 10 days after rotational shock. There is complete necrosis of this segment of the muscle, with calcification of residual muscle fibers, an area of intense inflammatory reaction, and a background of diffuse mononuclear cellular response. × 40.

FIG. 4. Section from the myocardium, showing an intramural coronary artery with a surrounding mononuclear cellular reaction. From an animal sacrificed 5 days after drum rotation. × 50.

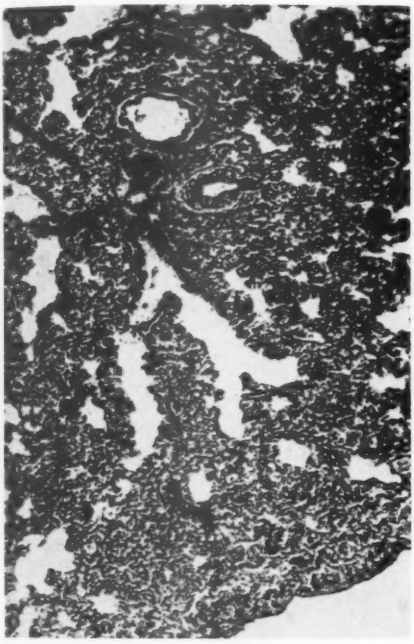

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FIG. 5. Section from the small intestine of a rabbit which died approximately 9 hours after drum rotation. There is almost complete ischemic necrosis of the segment in the lower portion of the photograph. Some viable glands are present in the segment in the upper half. × 40.

FIG. 6. Section from the fundus of the gallbladder which shows complete ischemic necrosis of this portion of the organ. From a rabbit that died approximately one hour after drum rotation. × 30.

FIG. 7. Section of gallbladder and adjacent liver from a rabbit which died approximately 2 hours after rotational shock. The edge of the liver comprising the gallbladder bed, as well as the gallbladder, show ischemic necrosis. × 30.

FIG. 8. Section from the gallbladder of a rabbit given a sympathomimetic amine in conjunction with drum rotation. Sacrificed 48 hours after rotation. Note complete necrosis of gallbladder with a dense accumulation of inflammatory cells within the wall and adjacent omental tissues. × 30.

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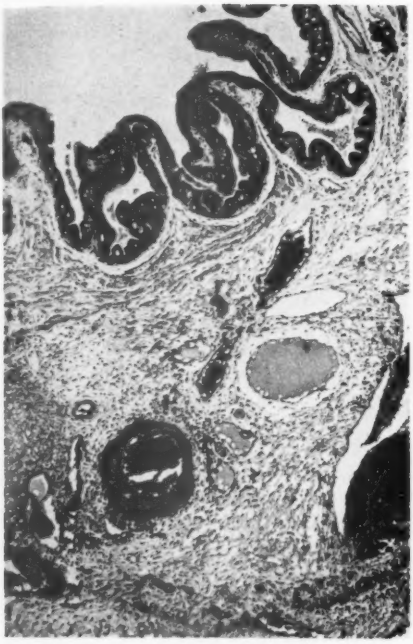
FIG. 9. Gallbladder section from a rabbit given a phenothiazine derivative and subjected to rotational shock. Sacrificed 48 hours following rotation. The epithelium appears intact, but there is marked edema of the gallbladder wall with minimal cellular infiltration. The vessels are patent, but one artery in the lower portion of the photograph shows a perivascular deposition of hyaline material. × 30.

FIG. 10. Section from the gallbladder of a rabbit sacrificed 10 days after drum rotation. Treated with a phenothiazine derivative prior to shock. Note fibrous thickening of gallbladder wall, absence of cellular inflammatory reaction, and apparent complete restoration of epithelium in the upper portion of the organ. × 30.

FIG. 11. Gallbladder section from a rabbit given a phenothiazine derivative in association with rotational shock. Sacrificed 10 days after rotation. The gallbladder had perforated into the surrounding omentum. Note papillary proliferation of epithelium, dense fibrous tissue thickening of the wall of the organ, but lack of cellular reaction. × 30.

FIG. 12. Gallbladder section from an animal treated with a sympathomimetic amine. Sacrificed 10 days after drum rotation. The gallbladder had perforated into the omental fat. There is epithelial hyperplasia and connective tissue proliferation in the wall of the gallbladder. The site of perforation is marked by a heavy accumulation of inflammatory cells and necrotic tissue. × 30.



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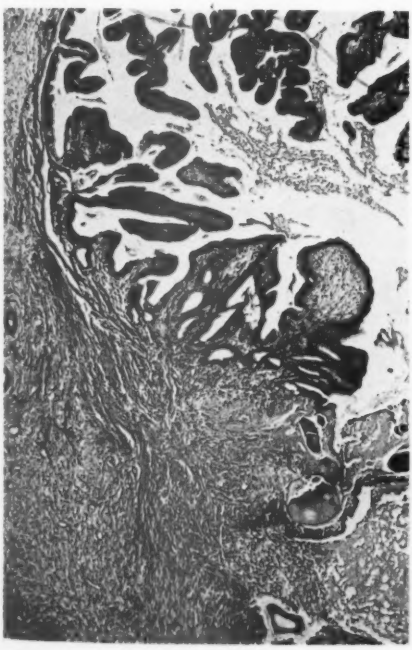

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PULMONARY HISTOLOGIC REACTIONS TO INHALED FIBERGLAS-PLASTIC DUST *

G. W. H. Schepers, M.D., D.Sc.†

Fiberglas-plastic‡ has in recent years gained such diversified and extensive commercial application that several thousands of workmen are currently handling this substance. In the manufacture of automobile bodies, airplane fuselages, boats, structural partitions, furniture, machine parts, etc., there are many operations involving sawing, planing, sanding or burnishing the plastic. Inadequate industrial hygiene may cause artisans to inhale dust generated through these processes.

There are scattered references to the mildly irritative cutaneous effects of polyester resin plastic,[1] of glass wool,[2] and of pulmonary reactions attributed to the inhalation of glass fibers.[3-7] It is of interest, therefore, to report the lesions provoked in experimental animals by dust derived from plastic-supported glass fibers.

Material and Methods

The biologic actions of two types of commercial fiberglas-plastic have already been reported,[8-11] and the experimental inhalation procedures have been outlined. The first variety contains a calcium-sulfate filler and is designated sheet fiberglas-plastic. The second type, produced as molded fiberglas-plastic, utilizes calcium carbonate as a filler. There are minor differences in the proportions of the glass fibers and polymerized resin, but chemically these ingredients are identical.

In the investigation with the first-mentioned substance, 20 rats, 64 guinea pigs and 9 rabbits were exposed for periods up to 9.3 months to aerosol concentrations of pulverized sheet fiberglas-plastic averaging 283 million particles per cubic foot of air. The experiment with the $CaCO_3$-filled molded fiberglas-plastic was conducted on 36 rats, 65 guinea pigs, 12 rabbits and 5 monkeys at an average aerosol concen-

* This study was co-sponsored by the Owens-Corning Fiberglas Corporation, Libbey-Owens-Ford Company, Pittsburgh Plate Glass Company (Fiber Division), Molded Fiber Glass Body Company, and the Office of Naval Research, Contract Nonr 1871(01). The inhalation exposures were completed at the Saranac Laboratory, Saranac Lake, N.Y.

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‡ The term "fiberglas-plastic" is an abbreviation for the more cumbersome trade name of the product "Fiberglas-reinforced polyester resin plastic." The material contains 3 ingredients: glass fibers (10 to 30 per cent), thermosetting polymerized polyester resin (30 to 60 per cent), and filler (20 to 50 per cent). Minute quantities of retardants and catalysts are added at various steps.

tration of 338 million particles per cubic foot of air for periods up to 25 months.

As controls to both experiments, 100 rats, 100 guinea pigs, 50 rabbits, and 10 monkeys remained unexposed to dust.

Histologic Observations

Rats

Sheet Fiberglas-plastic and Calcium Sulfate. No specific pulmonary lesion could be identified in this group although various minor reactions were elicited (Table I). The initial reaction was stronger than that found later. Moderate numbers of particle-filled macrophages were present both within the alveolar spaces and walls. Minor emphysema was noted in two animals. The alveolar ducts were slightly dilated in all the rats, and at isolated foci, phagocytes had congregated

Table I

Influence of Inhaled Particulate Fiberglas-plastic on the Rat Lung

	Experiment no. 1245*			Experiment no. 1259†				
Animal nos.:	201 202 203	206 207 208	223 224 225	153 154 155	157 158	159 169 170	172 173	160 161 162
Dust exposure (mos.):	3	6	9	3	6	9	12	15
Gross observations:								
Lung								
Subpleural pigmentation						+	++	+++
Focal atelectasis		xx				x	xx	x
Lymph node								
Hypertrophy				+	+	++	+	x
Induration						+	+	
Histologic features:								
Alveoli								
Granulomas								++
Macrophage catarrh	++	+		+	++	+	x	
Lipophage islets				++	++	++	+	
Mural infiltration	++	+		++	++	+		
Duct ectasia	+	+	x	+				
Hyperemia				++	+			
Emphysema	x	x						+
Lymphatics—macrophage infiltration								
Parenchymal follicles	+	x		x	xx	+		
Tracheobronchial nodes		+	x		+	++	++	++

* Sheet fiberglas-plastic + $CaSO_4$: Aerosol concentration, 283 million particles per cubic foot of air.

† Molded fiberglas-plastic + $CaCO_3$: Aerosol concentration, 338 million particles per cubic foot of air.

Key: + Slight reaction +++ Marked reaction
++ Moderate reaction x and xx Observations do not apply to all animals

in paraductal alveoli. Occasional dust-laden cells were found in the peribronchial lymphoid follicles, but there were no lesions in the tracheobronchial lymph nodes.

No deviations of a pathologic nature could be discovered in other organs.

Molded Fiberglas-plastic and Calcium Carbonate. In the lungs of the rats exposed to this substance, pathognomonic lesions were not definable, and the majority of the tissue reactions were transient. By the sixth month, groups of macrophages and lipophages had collected in subpleural and parabronchiolar sites, and there was associated hyperemia. Belatedly, slight emphysema and sparse focal granulomatous lesions appeared. Multinucleated, dust-filled macrophages infiltrated the cortical zones of regional lymph nodes.

No significant reactions were noted in organs other than the lungs.

Chemical Determinations. The limited tissue responses were matched by minor gravimetric changes in the lungs (Table II). The lung weights never exceeded those of the controls in the experiment with the $CaSO_4$-filled plastic, but in the $CaCO_3$ study the total tissue reaction was slightly greater, with appreciable augmentation of the lung weights

TABLE II

Inhalation of Particulate Fiberglas-plastic
*Chemical Determinations, Rat—Continuous Exposure**

	Experiment no. 1245†			Experiment no. 1259‡				
Animal nos.:	202 203	207 208	224 225	154 155	157 158	169 170	172 173	161 162
Exposure (mos.):	3	6	9	3	6	9	12	15
Lung weight (mg.)								
Fresh	2300	2200	1700	2800	3600	3000	3300	6100
Dried	177	285	232	423	521	339	558	931
Ash	9.6	10.6	9.5	13.8	20.4	19.3	23.6	41.1
SiO_2	0.08	0.08	0.14	0.6	0.9	0.9	0.8	1.3
Ratio (%)								
Ash/dried lung	3.44	3.64	3.38	3.27	3.88	5.7	4.45	4.42
SiO_2/ash	0.8	0.8	1.5	4.1	4.8	4.4	3.3	3.0
SiO_2/dried lung	0.03	0.03	0.05	0.14	0.18	0.25	0.15	0.14

* Chemical determinations were made by T. M. Durkan.

† Sheet fiberglas-plastic + $CaSO_4$: Aerosol concentration, 283 million particles per cubic foot.

‡ Molded fiberglas-plastic + $CaCO_3$: Aerosol concentration, 338 million particles per cubic foot.

after the ninth month. In the control rats the lung weights decreased 45 per cent beyond this point, so that the doubling of the weight at the end of 15 months of exposure to the fiberglas-plastic dust seems significant.

While the quantity of pulmonary silica increased steadily in both experiments as exposure continued, about 6 times as much silica accumulated in the lung in the experiment with the $CaCO_3$-filled fiberglas-plastic. The total lung ash increased significantly only in the latter experiment. This contrast was again reflected in the comparison of the SiO_2/lung-ash ratio.

Guinea Pigs

Exposure Phase. The responses in these animals were more clearly defined than in the rats, and the contrast between the lesions provoked in the experiments with the two types of fiberglas-plastic was sharper.

Sheet Fiberglas-plastic and Calcium Sulfate. The most impressive histologic features in the animals sacrificed at the end of 2 months of exposure to the $CaSO_4$-filled fiberglas-plastic dust were focal paraductal hyperemia and petechiae. At multiple points throughout the lung parenchyma, small accumulations of both agranular and dust-filled macrophages appeared. In a number of locations, macrophage infiltration of the alveolar walls also was present. Slight, diffuse, vesicular emphysema and dilatation of the alveolar ducts accompanied these alterations. The bronchioles and bronchi displayed some epithelial destruction. Among the desquamated cells, eosinophilic leukocytes were present in moderate numbers.

During subsequent phases, represented by animals sacrificed in groups of 4 at the end of 4, 5, 8, and 9.3 months' exposure, these tissue reactions regressed at variable rates as shown in Table III.

Molded Fiberglas-plastic and Calcium Carbonate. The reaction of the guinea pig lung to the $CaCO_3$-filled fiberglas-plastic was much more impressive. A highly characteristic pneumoconiotic lesion resulted in all animals. The tissue responses, though universal, were not uniformly distributed, being maximally concentrated in the basal lobes.

Two categories of reaction ensued. In one group it was transitory or regressive and included: hyperinflation, focal atelectasis, hyperemia, petechiae, desquamation of bronchiolar epithelium, parenchymal lymph follicle hyperplasia and macrophage infiltration. The occurrence of these lesions is summarized in Table III.

The second moiety of reactions had a retarded onset. These responses included: emphysema, koniophore cluster formation, and distortion and stenosis of bronchioles. These lesions were progressive. By far the most distinctive lesion is represented by the koniophore clusters illustrated in Figures 1 to 4. Each of the pneumoconiotic foci represented a more or less spherical unit of parabronchiolar lung tissue occupied by particle-filled multinucleated giant cells. In an average cross sec-

Influence of Inhaled Particulate Fiberglas-plastic on the Guinea Pig Lung; Exposure Phase

	Experiment no. 1245*					Experiment no. 1259†											
Animal no.:	1-4	5-8	9-12	13-16	17-20	1-4	5-8	9-12	13-16	17-20	21-24	25-28	29-32	33-36	37-40	41-46	146-152‡
Exposure (mos.):	2	4	6	8	9.3	2	4	6	8	10	12	15	18	21	24	25	25
Alveoli																	
Emphysema	+	x				+	x							+	+	+	+
Atelectasis (focal)						+	+	+	x	x	x						
Koniophore clusters									x	+	+	++	+++	+++	+++	+++	++++
Macrophage catarrh	++	+					x	x	+	+	++	++	+	x	x		
Hyperemia and petechiae	++					xx			x	x	xx	x	x	x	x		
Mural infiltration	xx	x	x	x	x	x	x	+	+	++	++	++	+	x			
Ducts and bronchioles																	
Stenosis and distortion							x	x	x	x	x	x	xx	++§	+++§	+++§	+++
Epithelial desquamation	+	x	x	x	x	+	++	++	xx	xx	xx	x	x				
Mucosal evagination														x	xx	xx	
Lymphoid tissue (Pulmonary)																	
Hyperplasia	x	+	+			+++	++	++	++	++	++	+	x				
Macrophage infiltration (Tracheobronchial)			x	x	x	+	+	+	x	x	x						
Hyperplasia		+	+	++	+	+	++	++	++	++	++	++	++	+	+	+	
Macrophage infiltration		+	++	++	+	+	+	+	++	+++	++	++	x	x			
Pigmentation										x	x	+	+	+			
Sinus catarrh						+	+	x	x	x	x	x	x	x	x		

* Sheet fiberglas-plastic + $CaSO_4$: Aerosol concentration, 283 million particles per cubic foot of air.

† Molded fiberglas-plastic + $CaCo_3$: Aerosol concentration, 338 million particles per cubic foot of air.

‡ This group of guinea pigs was exposed to an average aerosol concentration of 485 million particles per cubic foot of air.

Key: + Slight reaction
++ Moderate reaction
+++ Marked reaction
++++ Extreme reaction

x and xx Observations do not apply to all animals of group.

§ Adenomatoid reaction.

tion, up to 100 alveoli were involved. Many participating alveoli were overdistended, and some had become confluent through breakdown of alveolar walls. The sharp marginal definition of these units was particularly impressive. The virtually complete lack of stromal alterations such as deposition of elastic, reticulin, or collagen fibers in the alveolar septums between the koniophores was especially noteworthy. The size of the unit lesions proved to be proportionate to the duration and the intensity of dust exposure. In the group of guinea pigs exposed to the heaviest dust concentration for 25 months, some lesions attained a size up to 4 times those found at the same stage in animals which received lesser exposure. At the same time the individual macrophages were larger, and more breakdown of alveolar septums was manifest.

Toward the end of 21 months of exposure, epithelial evaginations from the bronchioles added to the complexity of the pneumoconiotic reaction (Fig. 5). Some of these sacs bulged among the surrounding koniophores; in cross section a rosette pattern was thereby created. These lesions are of interest because they correspond to similar responses induced experimentally by inhaled glass wool,[7] talc,[12] and asbestos fibers.[13]

Chemical Determinations. Gravimetric confirmation of the sequence of histologic changes was possible (Table IV). In the group of guinea pigs exposed to the $CaSO_4$ type of fiberglas-plastic, the lung weights agreed closely with those of normal animals except for terminal augmentation at the end of 9.3 months. In the guinea pigs which inhaled the $CaCO_3$-filled fiberglas-plastic, lung weights exceeded those of the controls by about 30 per cent. In the animals exposed to the $CaSO_4$-filled fiberglas-plastic dust, silica accumulated in the lung at about a third of the rate recorded in the animals which inhaled the $CaCO_3$-filled fiberglas-plastic dust. The higher value for the SiO_2/lung-ash ratio in the latter group of animals matched the pronounced histologic response.

Elimination Phase

Sheet Fiberglas-plastic and Calcium Sulfate. The transitory nature of the limited pulmonary responses in the guinea pig lung to this substance was clearly revealed when the animals were transferred to a normal air environment following 9 months of dust exposure. Immediately after cessation of dust exposure, there was a mildly intensified pulmonary reaction which subsided rapidly. The enhanced responses included catarrh of bronchial epithelium, focal hyperemia, petechiae and mural infiltration. The lesions regressed steadily after 2 months' residence in normal air. At the end of 6 months the alveolar walls were virtually normal. At no stage was any fibrosis detectable.

TABLE IV

Inhalation of Particulate Fiberglas-plastic
*Chemical Determinations, Guinea Pig—Exposure Phase**

	Experiment no. 1245†					Experiment no. 1259‡										
Animal no.:	3,4	7,8	11,12	15,16	17,18	3,4	7,8	11,12	15,16	19,20	23,24	27,28	31,32	35,36	39,40	45,46
Exposure (mos.):	2	4	6	8	9.3	2	4	6	8	10	12	15	18	21	24	25
Lung weight (mg.)																
Fresh	5500	5900	6300	6600	8600	6300	7000	7100	7700	8200	8600	9200	9400	10400	11700	9000
Dried	786	781	847	933	1105	868	931	988	1167	1255	1461	1334	1384	1223	1310	1313
Ash	30.2	30.1	32.6	34.8	42.3	34.1	35.6	38.5	45.0	48.8	57.2	49.3	61.5	51.1	57.0	58.1
SiO_2	0.3	0.3	0.4	0.5	0.8	0.6	0.8	1.5	2.2	2.5	2.7	2.4	2.6	3.0	3.1	3.3
Ratios (%)																
Ash/dried lung	3.84	3.85	3.82	3.72	3.84	3.97	3.83	3.88	3.85	3.87	3.92	3.71	4.41	4.17	4.2	4.42
SiO_2/ash	0.9	1.0	1.4	1.2	1.8	1.8	2.1	3.9	4.9	5.1	4.8	4.8	4.3	5.9	5.8	5.7
SiO_2/dried lung	0.04	0.04	0.06	0.05	0.07	0.07	0.08	0.15	0.19	0.2	0.19	0.18	0.19	0.24	0.25	0.25

* Chemical determinations were made by T. M. Durkan.
† Sheet fiberglas-plastic + $CaSO_4$: Aerosol concentration, 283 million particles per cubic foot of air.
‡ Molded fiberglas-plastic + $CaCO_3$: Aerosol concentration, 338 million particles per cubic foot of air.

Molded Fiberglas-plastic and Calcium Carbonate. With the exception of emphysema, which advanced slowly (Table V), all lesions which had been established at the end of 24 months speedily regressed after the animals were returned to normal air. One of the first alterations consisted of evacuation of phagocytes from the alveolar spaces. This commenced within the first week and was virtually complete by the end of the second. Later a few macrophages reappeared in the alveolar spaces. These carried no obvious dust load. By the end of the second week the bronchiolar lumens had been cleared of cellular detritus, and and some regeneration of epithelium was evident.

After 2 months in a normal air environment, resolution of the pneumoconiotic unit lesions began by breaking up of the koniophore syncytiums into their component elements. Some of the resultant unicellular macrophages found their way into the alveolar walls. The majority escaped from the lung via the bronchial passages (Fig. 6). The markedly disorganized bronchiolar mucosa rapidly returned to normal except at a few levels where evaginations of the epithelium persisted in relation to residual koniophore clusters. In the tracheobronchial lymph nodes a moderately marked macrophage infiltration of the medulla developed.

Chemical Determinations. The foregoing regressive changes were matched by reversal of the silica, lung-ash and lung-weight values (Table VI). As the SiO_2 concentration had not attained unusually high levels in the experiment with the aerosolized $CaSO_4$-filled sheet fiberglas-plastic, the least change was noted in this series. At the end of the 3 months' elimination period in the study with the $CaCO_3$-filled fiberglas-plastic, there was decisive reduction of the SiO_2 and lung-ash values. The retarded release of the SiO_2 favors the interpretation that this substance existed primarily within the koniophore clusters.

Rabbits

Sheet Fiberglas-plastic and Calcium Sulfate. The response to the inhaled dust was exceedingly limited (Table VII). In the animals sacrificed at the end of the third, sixth and tenth months, the only perceptible deviations were focal capillary dilatation and moderate alveolar macrophage catarrh. The lymph nodes, respiratory passages, blood vessels and extrathoracic organs were normal. Virtually no abnormalities were detected in the lungs of the animals sacrificed 3 and 6 months after transfer to normal air.

Molded Fiberglas-plastic and Calcium Carbonate. The histologic responses were also limited in scope in this group of rabbits. The chief reactions were hyperemia, koniophore infiltration into some of the

TABLE V

Influence of Inhaled Particulate Fiberglas-plastic on the Guinea Pig Lung; Elimination Phase

	Experiment no. 1245*							Experiment no. 1259†					
Animal no.:	21-36	37-40	41-44	45-48	49-52	53-56	57-60	47-48	49-50	51-52	53-54	56-60	
Normal air (days):	1-7	14	21	30	60	120	180	8	14	30	60	90	
Alveoli													
Emphysema	xx	x	x						+	+	++	++	
Koniophore clusters								+++	+++	+++	++	+	
Macrophage catarrh									+	++	++	++	
Mural infiltration	x	xx	xx	xx	x					+	+		
Epithelial desquamation	x	+	++	++	+	x							
Ducts and bronchioles													
Stenosis and distortion								++	+	+			
Mucosal evagination								xx	xx	x	x	x	
Adenomatoid changes								xxx	xxx	xx	x	x	
Lymph nodes													
Hyperplasia	x												
Macrophage infiltration	+	x	x	x	x			++	++	++	++	++	

* Normal air after 9 months' exposure to sheet fiberglas-plastic + $CaSO_4$.
† Normal air after 24 months' exposure to molded fiberglas-plastic + $CaCO_3$.
Key: + Slight reaction +++ Marked reaction
++ Moderate reaction x, xx and xxx Observations do not apply to all animals.

TABLE VI

Inhalation of Particulate Fiberglas-plastic
*Chemical Determinations, Guinea Pig—Elimination Phase**

	Experiment no. 1245†								Experiment no. 1259‡					
Animal no.:	17,8	21-34	37,38	41,42	45,46	49,50	53,54	57,58	39,40	47,48	49,50	51,52	53,54	56-60
Normal air (days):	0	1-7	14	21	30	60	120	180	0	8	14	30	60	90
Lung weight (mg.)														
Fresh	8600	9400	8700	8600	8400	7600	6900	6900	9200	9500	9800	10000	9800	9300
Dried	1105	1266	1143	1210	1042	1145	1011	980	1310	1481	1575	1668	1535	1346
Ash	42.3	49.1	44.7	46.6	40.1	44.1	35.2	34.9	57	66	64.7	62.6	62.8	53
SiO_2	0.8	0.8	0.7	0.6	0.6	0.5	0.5	0.4	3.1	3.3	3.9	3	3.1	2.6
Ratios (%)														
Ash/dried lung	3.84	3.79	3.59	4.28	3.89	3.85	3.54	3.54	4.2	4.45	4.11	3.75	4.06	3.61
SiO_2/ash	1.8	1.6	1.7	1.1	1.5	1	1.3	1.4	5.9	5	6	4.9	5	5
SiO_2/dried lung	0.07	0.06	0.06	0.05	0.06	0.05	0.05	0.05	0.25	0.23	0.25	0.18	0.2	0.19

* Chemical determinations were made by T. M. Durkan.
† Normal air after 9 months' exposure to sheet fiberglas-plastic + $CaSO_4$.
‡ Normal air after 24 months' exposure to molded fiberglas-plastic + $CaCO_3$.

TABLE VII

Influence of Inhaled Particulate Fiberglas-plastic on the Rabbit Lung

	Experiment no. 1245*					Experiment no. 1259†							
Animal no.:	236	231	232, 233 234, 238	237	239	224	187	251	252	221	188	189	190
Dust exposure (mos.):	3	6	9	9	9	4	6	7	8	9	12	18	24
Normal air (mos.):				3	6								
Alveoli													
Subpleural pigmentation					++			+	+++	+++	+	+	++
Hyperemia	+		x			++	++	++	+	++	+		
Macrophage catarrh	++	+	++			++	+	+	+	+	+	+	
Mural infiltration						+++	+	+	+	++	+	+	
Duct ectasia											+	+	+
Lymph follicle infiltration						+	+	++	+			++	++

* Sheet fiberglas-plastic + $CaSO_4$: Aerosol concentration, 283 million particles per cubic foot of air.

† Molded fiberglas-plastic + $CaCO_3$: Aerosol concentration, 338 million particles per cubic foot of air.

Key: + Slight reaction +++ Marked reaction
++ Moderate reaction x Observations do not apply to all animals of group.

pulmonary lymphatic channels and minor distention of alveolar ducts with corresponding terminal partial collapse of adjacent alveolar spaces. The epithelium of the respiratory tract remained unaffected during the full 24-month period of dust exposure.

Monkeys

Of the 4 varieties of animals investigated, the histologic reactions in the monkey were probably the most significant. Dust lesions were distributed throughout the lungs, but especially in the basal lobes (Table VIII). While almost no cells were discernible in the alveoli, there was marked invasion of the alveolar walls by particle-filled

TABLE VIII

Influence of Inhaled Particulate Fiberglas-plastic on the Monkey Lung

	Experiment no. 1305				
Animal no.:	2	1	3*	4†	5
Exposure (mos.):	6	8	8	8	8
Distribution of lesions					
Apexes	+	+++	+++	++	+
Bases	++	+++	+++	++	+
Alveoli					
Emphysema	+	+	++	+	++
Macrophage infiltration	++	+++	+++	+++	+
Capillary occlusion		+	++	++	
Ducts					
Mural infiltration	+++	+++	+++	+++	+
Dilatation	++	++	++	++	+
Distortion	+	++	++	+	+
Bronchioles					
Stenosis	+	++	++	+	
Macrophage infiltration	+++	++++	++++	+++	+
Parabronchial koniophore deposits	++	+++	+++	++	+
Blood vessels					
Stenosis	+	++	+	+	
Adventitial infiltration	++++	++++	++++	++++	++
Koniophore infiltration	+++	+++	++++	++	+
Tracheobronchial lymph nodes					
Medullary hyperplasia			++	+	+
Macrophage infiltration	+	+	+	++	+
Sinus catarrh			++	+	
Endothelioid cell hyperplasia	+	++	+	++	++

* Foci of pneumonia present, unrelated to experimental lesions.

† Animal received 150,000 units of penicillin for *Staphylococcus albus* abscess of ear.

Key: + Slight reaction +++ Marked reaction
++ Moderate reaction ++++ Extreme reaction

macrophages. This infiltration was accompanied by capillary occlusion. Phagocytes also were present in the walls of the alveolar ducts, and the thickening thus caused, plus distention of the affected alveolar ducts and associated vesicular emphysema, resulted in appreciable derangement of the pulmonary architecture (Fig. 8). There also was marked macrophage infiltration of the peribronchiolar lymphatic spaces so that many of the bronchioles were surrounded by broad cellular collars. The majority of the pulmonary blood vessels were also extensively ensheathed by thick cuffs of particle-filled phagocytes.

Although no hyperplasia of lymphoid tissue occurred, the dust-laden macrophages infiltrated densely around some of the intrapulmonary lymphoid follicles. In the tracheobronchial lymph nodes some macrophage infiltration and a slight to moderate degree of intrinsic hyperplasia occurred. No dust-provoked abnormality could be discerned in the extrathoracic organs.

At the end of the eighth month of fiberglas-plastic dust exposure, no collagen had formed and no fibrocytes were in evidence in any of the sites where macrophages had infiltrated, nor was there any indication of degeneration of the cells harboring the dust particles.

Comment

The diversity of reactions evoked in the 4 species of experimental animals leaves a measure of uncertainty as to which should be extrapolated to man. The human lesion induced by quartz dust is a synthesis of the types of reactions found in rats, guinea pigs and rabbits.[16] Quite possibly, therefore, at least some and perhaps all of the lesions found in the present study with the fiberglas-plastic dusts may be recapitulated in human experience. It is to be hoped that the lungs of industrial workers unavoidably exposed to the fiberglas-plastic dust will not react more severely or more permanently than did those of the experimental animals.

Radiographic investigation was, unfortunately, carried out only on the rabbits, and in these animals the tissue responses were minimal. Judging by the reaction in the monkey and in the guinea pig, in which some lesions were more than 3 mm. in cross section, radiographic micro-mottling should be anticipated in man. In view of the reversibility of the animal lesions and the lack of a fibrotic component, it would be prudent, however, not to reach a medico-legal verdict based purely on roentgenographic criteria, as long as active dust exposure continues. Cessation of work may lead to resolution of pulmonary mottling.

It is possible that the human lung may respond, as did the guinea pig lung, by involvement of bronchioles and alveolar ducts. This might produce clinical symptoms. In view of the fact that the guinea pig bronchiolar lesions cleared up after the animals were removed from the dust environment, it is to be hoped that symptoms will prove equally reversible in man.

While no pulmonary hypertension was recorded for rabbits, as these animals did not develop perivascular pulmonary lesions, the impediment to the vascular system noted in monkeys may find a counterpart in pulmonary hypertension in human subjects. In view of this possibility, the electrocardiograph might advisedly rank as one of the basic tools in controlling the health of persons unavoidably exposed to the fiberglas-plastic dust.

The differential influence of the $CaSO_4$ and $CaCO_3$ fillers remains unexplained. Evidently, the addition of calcium sulfate either facilitates the re-elimination of the inhaled fiberglas-plastic particles or retards their retention. The limitation of the pneumoconiotic reaction by the $CaSO_4$ may perhaps also be an antidotal effect. Thus, it has been shown that calcined gypsum, which is largely $CaSO_4$, has a decisive inhibitory effect on inhaled quartz dust.[17] The $CaSO_4$ partly counteracts the retention of SiO_2 in the lung and partly neutralizes its pathogenic action.

Summary

1. Inhaled fiberglas-plastic dust provoked a very limited, reversible, nonspecific pulmonary reaction in rats. Substitution of $CaSO_4$ for $CaCO_3$ as the filler reduced the effect of the inhaled dust.

2. Exposure of guinea pigs to $CaCO_3$-filled molded fiberglas-plastic induced a characteristic pneumoconiotic lesion which did not, however, include a fibrotic component. In addition, significant involvement of the bronchioles and alveolar ducts occurred. All these reactions were reversed on cessation of dust inhalation. The pulmonary responses to $CaSO_4$-filled sheet fiberglas-plastic were comparatively insignificant.

3. The rabbit lung proved relatively resistant to both varieties of inhaled fiberglas-plastic dust.

4. In the monkey, marked peribronchiolar, perivascular and perilymphatic cellular infiltrations occurred within 6 months after exposure to the $CaCO_3$-filled molded fiberglas-plastic dust.

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[*Illustrations follow*]

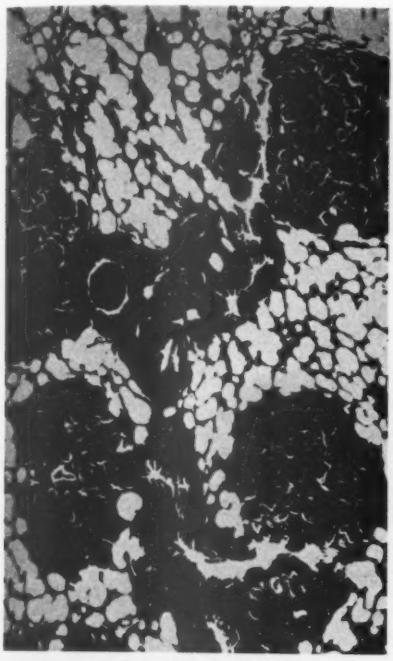
LEGENDS FOR FIGURES

FIG. 1. Guinea pig lung. Exposure 25 months to fiberglas-plastic dust at an average particle concentration of 338 million per cubic foot of air. Parabronchiolar alveoli are filled with koniophores. Hematoxylin and eosin stain. × 35.

FIG. 2. Guinea pig. Considerably larger lesion induced by a heavier concentration of fiberglas-plastic dust. Exposure 25 months to 485 million particles per cubic foot of air. Hematoxylin and eosin stain. × 8.

FIG. 3. Guinea pig; 25 months' exposure. Pseudosyncytial intra-alveolar multinucleated koniophores, exhibiting no cytoplasmic or nuclear degeneration as a result of the ingestion of fiberglas-plastic dust particles. Hematoxylin and eosin stain. × 350.

FIG. 4. Guinea pig; 25 months' exposure. Limited reticulin formation in the alveolar walls. Very few macrophages have penetrated. The spaces around the intra-alveolar giant cells are artifacts. Bielschowsky-Foot stain. × 350.



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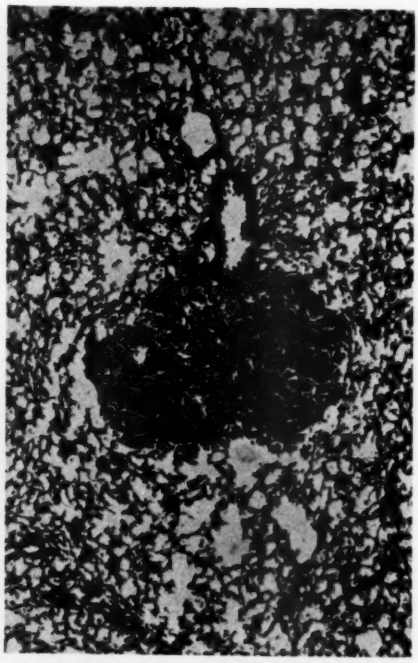

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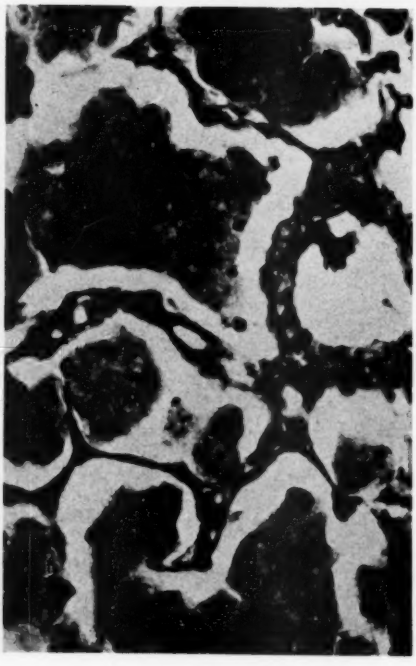

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FIG. 5. Guinea pig; 21 months' exposure. Proliferation and evagination of bronchiolar epithelium in relation to a focal collection of fused intra-alveolar koniophores. Mallory trichrome stain. × 260.

FIG. 6. Guinea pig; resided in normal air for 3 months following 24 months' continuous exposure to fiberglas-plastic dust. Resolution of pneumoconiotic foci by fragmentation of pseudosyncytial intra-alveolar koniophore clusters. The component macrophages are escaping via the respiratory passages. Hematoxylin and eosin stain. × 260.

FIG. 7. Monkey lung; fiberglas-plastic dust exposure 6 months. Congregation of dust-filled macrophages in peribronchiolar, periductal and perivascular zones. Hematoxylin and eosin stain. × 35.

FIG. 8. Monkey; 6 months' exposure. A broad zone of particle-laden macrophages has infiltrated into the cortex of a parenchymal lymph follicle without associated lymphoid hyperplasia. Hematoxylin and eosin stain. × 260.

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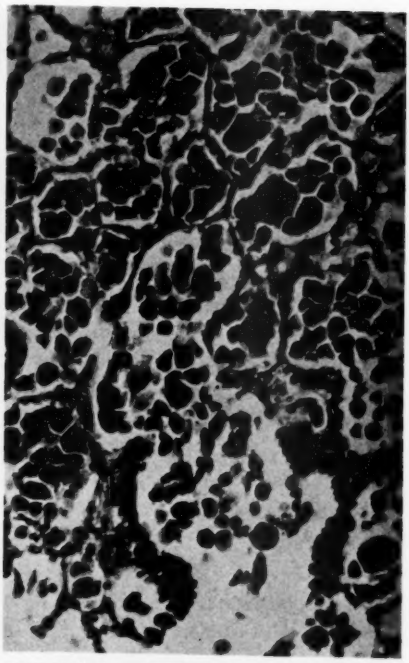

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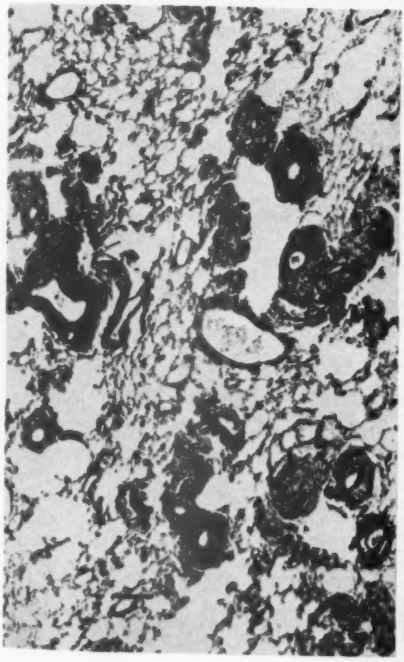

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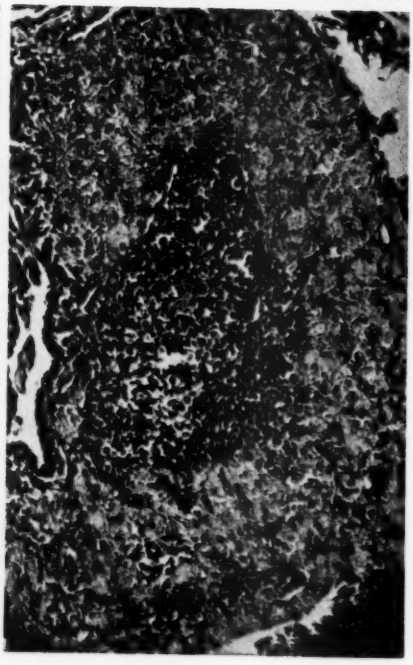

8

ENZYMATIC STAINING REACTIONS IN THE KIDNEYS OF POTASSIUM-DEPLETED RATS *

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The application of various histochemical enzyme techniques to the mammalian kidney has revealed distinctive and reproducible staining patterns within the various portions of the nephron. It is not surprising that most reactions in the cortex are positive, particularly in the proximal convoluted tubules, since the maximum work load of the kidney is performed in these segments. Some enzymatic reactions are also quite distinct in the thin limbs of Henle's loops and the collecting ducts within the medulla.[1]

In potassium deficiency, there occurs an increase in kidney weight, mainly due to hyperplasia of the medulla. Such kidneys have been examined by conventional methods[2-10] and more recently by the microdissection technique.[11] The most outstanding alterations occur in the collecting tubules. Other portions of the nephron reveal less consistent and more variable alterations. These are of the severest degree when weanling rats are given the deficient diet.[12]

Histochemical enzyme staining techniques have been applied to potassium-deficient kidneys by Spargo,[7] Craig and Schwartz,[9] and by Pearse and Macpherson.[13] The results reported have been somewhat contradictory. We have, therefore, investigated a number of histochemical staining reactions in the kidneys of potassium-deficient rats and selected those which demonstrate enzymatic activity in the medullary structures of the normal kidney. An attempt has been made to correlate the remarkable variations in histochemical reactions in the renal medulla with certain functional alterations known to occur in the potassium-deficient kidney.

MATERIAL AND METHODS

Young rats of the Wistar strain, weighing 150 to 200 gm., were used throughout. Thirty rats were fed a potassium-deficient diet and sacrificed after 15 to 25 days. An additional 20 rats were placed initially on a protein-deficient diet supplemented by all the necessary vitamins, as suggested by Spargo.[7] After the initial weights had been reduced 25 per cent, the rats were given a synthetic diet deficient in both potassium

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and sodium. Our experience was in agreement with that of Craig and Schwartz[9] who observed an enhancement of tissue alterations induced in potassium-depleted rats also receiving a low sodium intake.

Following sacrifice, pieces of kidney and heart were fixed in 10 per cent formalin and Rossman's fluid. Sections were stained with hematoxylin and eosin, the periodic acid-Schiff (PAS) technique and occasionally by the Jones silver-methenamine stain.[14]

Histochemical staining was carried out on free-floating, unfixed frozen sections and on sections prepared from thin tissue blocks fixed overnight in cold Baker's formalin. The following methods were used: alkaline phosphatase in formalin-fixed frozen sections according to Gomori (incubation period, 5 to 15 minutes), acid phosphatase in a modification of Gomori's technique using a pH of 6, preferably on formalin-fixed frozen sections (incubation period, 15 to 60 minutes)[15] and adenosine triphosphatase with the technique of Wachstein and Meisel (incubation period, 5 to 15 minutes).[16] For succinic dehydrogenase, unfixed frozen sections were incubated for 5 to 30 minutes in a mixture which contained sodium succinate and Nitro-BT [2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride].[17] In earlier experiments, we used the technique of Farber, Sternberg and Dunlap[18] for the demonstration of diphosphopyridine nucleotide (DPN) diaphorase in unfixed frozen sections. Later, the modification of Novikoff and Masek was substituted.[19] Formalin-fixed frozen sections were incubated in a mixture containing Nitro-BT as an indicator and, in addition, reduced diphosphopyridine nucleotide (DPNH) and the buffer (incubation time, 5 to 30 minutes). In some instances the results were checked by the use of fresh frozen sections.

For the demonstration of triphosphopyridine nucleotide (TPN) diaphorase, both the original technique of Farber and co-workers,[18] as well as the modified procedure of Nachlas, Walker and Seligman,[20] were used on fresh frozen sections (incubation time, 15 to 20 minutes). In addition, an incubation mixture which contained only TPNH, Nitro-BT and the buffer was used with both fixed and unfixed frozen sections.

In most instances a normal control rat was sacrificed simultaneously with the experimental rat in order to evaluate possible variations in the staining reactions.

Results

Our observations in the kidneys of potassium-deficient rats were similar to those described by various investigators and may be summarized as follows: The collecting tubules in the outer zone of the

medulla showed marked swelling of the cytoplasm and increase in the number of cells with apparent obstruction of the lumens. In the segments of excretory ducts located in the outer medulla there was a marked increase in the dark-staining intercalated cells, as has been pointed out by Oliver and co-workers.[11] The lumens of collecting ducts located in the cortex were often dilated. The collecting ducts close to the papilla showed a fairly normal configuration but were distended by innumerable eosinophilic granules which gave a positive PAS reaction.[7] There was also an increase in PAS staining in other structures of the medulla. Basement membranes stained more strongly, and an increased number of mononuclear cells containing PAS-positive material was seen. The cells in the thin limbs of Henle's loops often had an increased amount of cytoplasm and contained PAS-positive material. The broad ascending limbs of Henle's loops and the distal convoluted tubules appeared essentially normal although they were occasionally compressed by dilated collecting ducts. All structures that stained with the PAS technique also gave a positive staining reaction with the silver-methenamine stain (Fig. 1). Within the cortex, occasional proximal convoluted tubules showed swelling of cells as well as focal atrophy. The atrophy increased with the duration of the potassium deficiency and was more marked in the kidneys of experimental animals after 25 days as compared to those after 15 days.

Histochemical Staining Reactions

Alkaline Phosphatase. There was focal diminution of the staining reaction in proximal convoluted tubules corresponding to the focal atrophy observed in conventional sections. Granules in the collecting tubules did not stain but were clearly recognized by their refractility.

Acid Phosphatase. With the use of a modification of Gomori's method at pH 6.0 in formalin-fixed frozen sections, a consistent staining reaction was noted in the proximal convoluted tubules. Lead deposits indicative of enzyme activity appeared as granules varying from 1 to 3 μ in diameter. The straight or distal portions of the convoluted tubules contained fewer droplets and exhibited some staining of the brush borders. If the incubation period was not extended beyond 30 to 60 minutes, only faint nuclear staining was noted. The ascending broad limbs of Henle's loops exhibited a moderate reaction, as evidenced by the deposit of fine, dust-like granules. A slight although irregular reaction also appeared in the collecting ducts, thin limbs of Henle's loops, and occasional interstitial cells (Fig. 3). In unfixed sections, the lead sulfide deposit appeared in a more diffuse, dust-like fashion in the cortical tubules. Deposits of larger granules

were not seen. Both the ascending limbs of Henle's loops and the collecting ducts revealed a fairly strong reaction after 15 minutes' incubation. After longer incubation, nuclear staining became very marked in unfixed sections.

In the experimental animals there was considerable increase in the overall staining of the medulla; this could be recognized by naked-eye inspection of the sections. The increase was due to the presence of abnormal granules and an actual increase in activity in several medullary structures. This was the case in the collecting ducts, particularly in the segments located close to the papilla. Acid phosphatase activity was less marked in segments located in the outer medulla. There was also a striking increase of staining in the cells which formed the thin limbs of Henle's loops (Fig. 4) and in interstitial stromal cells, particularly near the papilla. In the region of the corticomedullary junction, there was a reduction of the reaction in the ascending limbs of Henle's loops; this was most clearly recognized in unfixed tissue, but was also seen in formalin-fixed frozen sections. Within the cortex, occasional atrophic proximal convoluted tubules showed reduced staining, as in the case of alkaline phosphatase.

Nonspecific Esterase. With naphthol AS as a substrate, there was a marked difference in the staining pattern in fixed and unfixed sections of the normal kidney. In unfixed sections, deposits were diffuse and dust-like in the proximal convoluted tubules. Glomeruli did not react and only slight staining was evident in the ascending limbs of Henle's loops. In the medulla there was rather intense and regular staining in the collecting ducts but only faint and somewhat variable staining in the thin limbs of Henle's loops. In fixed sections, the overall staining was more intense except in the collecting ducts. Proximal convoluted tubules of the cortex contained cells with both fine, dust-like granules and larger granules measuring 1 to 3 μ in diameter. In some cortical structures, obviously the distal convoluted tubules and initial portions of the collecting ducts, esterase activity was limited to isolated scattered cells. There was a slight reaction in cells of Bowman's capsule and in an occasional glomerular epithelial element. Staining in the ascending limbs of Henle's loops was moderately strong (Fig. 5). The thin limbs also exhibited distinct activity, but the collecting ducts stained only weakly (Fig. 7). The epithelium covering the renal papillas was also active. In addition, occasional stromal cells reacted in positive fashion.

In experimental animals, a reduction of enzyme activity was apparent in some of the proximal convoluted tubules. As in acid phos-

phatase preparations, the ascending limbs of Henle's loops at the corticomedullary junction showed a reduced staining reaction (Fig. 6). The collecting ducts of the medulla exhibited increased staining in segments close to the papilla, and abnormal granules participated in the reaction. There was a most striking increase in esterase activity in the thin limb of Henle's loop; this extended through its entire extent (Fig. 8). Interstitial cells, particularly those located close to the renal pelvis, were likewise the seat of striking esterase activity. An additional feature was the presence of an increased amount of bluish-staining protein in medullary capillaries. It is interesting that all of these phenomena were much more distinct in formalin-fixed than in fresh frozen tissue.

Adenosine Triphosphatase. The normal distribution of adenosine triphosphatase in the rat kidney has been described previously.[21,22] In formalin-fixed frozen sections, activity occurred in glomeruli, capillaries, ascending limbs of Henle's loops and distal convoluted tubules. The proximal convoluted tubules exhibited less activity, and this appeared mainly in brush borders. In the medulla, the inner cell borders of the collecting ducts were stained after 5 to 10 minutes (Fig. 9). Medullary capillaries reacted only after somewhat longer incubation periods.

In the potassium-deficient animals, there was a significant increase in the staining of the outer borders of cells in the collecting ducts, best seen in sections incubated for 5 to 10 minutes (Fig. 10). There was also a moderate reduction in the staining intensity of ascending limbs of Henle's loops and the distal convoluted tubules.

Succinic Dehydrogenase. When Nitro-BT was used instead of neotetrazolium, intense staining was seen within only a few minutes of incubation. Maximum activity was noted in the proximal portions of the proximal convoluted tubules and in the ascending limbs of Henle's loops. If staining was prolonged (15 to 30 minutes), very distinct activity was also seen in collecting ducts, mainly in the outer, and to a lesser degree, in the inner medulla. Within the collecting ducts in the outer medulla, occasional cells showed a very strong reaction. These obviously corresponded to the dark-staining intercalated cells. The thin limbs of Henle's loops reacted only very faintly.

In the potassium-depleted animals, there was a depression of activity in atrophic tubules in the cortex and a distinct although slight overall reduction in the ascending limbs of Henle's loops and in the distal convoluted tubules. The reaction in collecting ducts appeared to be somewhat enhanced, and a larger number of dark-staining intercalated

cells was seen. The thin limbs of Henle's loops stained faintly, but an unequivocal increase in the staining reaction as compared with the control animals could not be detected.

DPN Diaphorase. The normal distribution patterns of DPN diaphorase in unfixed frozen sections has been described previously.[23] In formalin-fixed sections, practically all renal cells, including those of the glomerulus, the thin limbs of Henle's loops, and vessel walls, show activity. In the cortex, the ascending limbs of Henle's loops and the distal convoluted tubules show a somewhat stronger staining than do the proximal convoluted tubules. Formazan deposits indicating enzymatic activity were very sharply localized in the mitochondria. With formalin fixation, however, there was some reduction in staining in the thin limbs of Henle's loops. The intensely staining intercalated cells of the collecting ducts were particularly prominent (Fig. 11).

In the potassium-depleted animals, there was a reduction in staining of some proximal as well as distal convoluted tubules. There was a moderate overall reduction of activity in the ascending limbs of Henle's loops. A larger number of strongly staining cells appeared in the median portions of the collecting ducts; this corresponded to the increase in intercalated cells in potassium deficiency (Fig. 12). There was also a somewhat stronger reaction in collecting ducts in the inner medulla. The atypical granules reacted in positive manner. The thin limbs of Henle's loops exhibited a slight increase in staining, as did some interstitial cells.

TPN Diaphorase. When the original method of Farber and coworkers[18] was used, staining was often spotty. Better and more consistent results were obtained with the technique of Nachlas and his associates.[20] The best results, however, were obtained with formalin-fixed sections when TPNH was used in the incubation mixture. Strongest activity was observed in the proximal convoluted tubules, the ascending limbs of Henle's loops, and in the collecting ducts of the medulla. The distal convoluted tubules stained less intensely with the exception of the macula densa, which could be clearly recognized by its deeper staining.[20] Glomeruli showed only weak activity, but the thin limbs of Henle's loops stained distinctly, particularly in unfixed sections.

In the kidneys of experimental animals, there was increased TPN diaphorase activity in the collecting ducts. There was also a slight increase in the thin limbs of Henle's loops, and a moderate decrease in the ascending limbs of Henle's loops and in those parts of the cortical tubules that had become atrophic. Staining of the macula densa was similar to that of normal controls (Fig. 2).

Discussion

Many histochemical enzyme staining reactions are regularly reproducible in the mammalian kidney and are, by now, well established.[1] Several observations made in this study, however, deserve comment (Table I). With a modified technique for acid phosphatase, the lead

Table I

Histochemical Renal Alterations in Potassium-Deficient Rats

Enzyme reaction	Changes observed
Alkaline phosphatase	Focal diminution in atrophic proximal convoluted tubules.
Acid phosphatase	Focal diminution in atrophic proximal convoluted tubules. Reduction in ascending limbs of Henle's loops. Marked increase in collecting ducts near papilla, less in outer medulla. Marked increase in thin limbs of Henle's loops and in interstitial stromal cells of medulla.
Nonspecific esterase	Similar to acid phosphatase.
Adenosine triphosphatase	Moderate reduction in ascending limbs of Henle's loops and distal convoluted tubules. Increase in outer cell borders of collecting ducts.
Succinic dehydrogenase	Reduction in atrophic tubules of cortex. Slight reduction in ascending limbs of Henle's loops and in collecting ducts.
DPN diaphorase	Reduction in atrophic proximal convoluted tubules. Moderate reduction in ascending limbs and distal convoluted tubules. Moderate increase in collecting ducts near papilla and greater increase in collecting ducts in the outer medulla. Slight increase in thin limbs of Henle's loops and interstitial cells.
TPN diaphorase	Similar to DPN diaphorase although somewhat less marked.

sulfide deposits indicating enzymatic activity were deposited in the rat kidney as coarse (1 to 3 μ) granules. A similar distribution pattern was found with various azo dye techniques for acid phosphatase[24,25] and for esterase.[25] It should be pointed out, however, that in unfixed frozen sections, such a staining pattern was not apparent. In such preparations, stain deposits occurred as tiny, evenly distributed cytoplasmic granules. It is worth noting that in the kidneys of other species —for instance, the rabbit—acid phosphatase activity is not characterized by the appearance of coarse granules in formalin-fixed sections.[15]

In preparations stained for nonspecific esterase, there were also differences in the distribution patterns in formalin-fixed and unfixed frozen sections. It was particularly noticeable that in formalin-fixed sections, activity in the collecting ducts was suppressed, while in the thin limbs of Henle's loops there was significant staining. In some cortical tubules, obviously the distal convolutions and initial portions of the collecting ducts, only scattered cells gave a positive staining reaction. These were first described by Hess and Pearse[26] using *o*-acetyl-5-bromoindoxyl as substrate. On the basis of studies utilizing

special inhibitors, these investigators considered the reaction to be due to an esterase with cathepsin-like activity. Further investigation will be necessary to substantiate this assumption. The physiologic significance of these cells, which are indistinguishable by conventional techniques, is unknown.

With the techniques for dehydrogenase and DPN diaphorase, and less regularly with the TPN diaphorase technique, dark-staining intercalated cells can be demonstrated readily in the median portions of the collecting ducts[27,28] by their strong staining. The function of these peculiar cells is also unknown at present.

The use of formalin fixation, first advocated by Novikoff and Masek,[19] permits a marked improvement in the techniques designed to demonstrate DPN and TPN diaphorase activity. The simplification of the incubation mixtures, replacing various substrates by DPNH and TPNH and the introduction of Nitro-BT, has contributed to the ease of performance of these techniques. The distribution patterns are essentially similar to those first described by Farber and his co-workers[18] and later confirmed, though somewhat modified, by others. In the case of the kidney, the improved techniques permit definite localization of formazan deposits in mitochondria, as well as differential staining of the macula densa.

Alterations of enzymatic staining reactions in experimental animals were most regularly noted in the medulla. By far the greatest increase of staining was observed with the acid phosphatase and esterase techniques. Obviously the increase occurred in 3 different structural units: the excretory ducts, the thin limbs of Henle's loops, and the interstitial cells. It should be emphasized that alterations in the thin limbs of Henle's loops can be recognized only with difficulty, if at all, in conventionally stained sections. The peculiar granules deposited in collecting ducts reacted with the staining techniques for acid phosphatase, esterase, and DPN and TPN diaphorase. In general, there was considerably less marked increase in activity of oxidative enzymes. However, many more cells in the collecting ducts of the outer medulla gave the strong staining reaction which seemed to be associated with intercalated cells. This is in agreement with the increased number of these cells observed in the kidney in potassium deficiency, described by Oliver and co-workers.[11]

A significant increase of adenosine triphosphatase activity was encountered in the outer membranes of collecting ducts in the potassium-deficient experimental animals. It should be recalled that adenosine triphosphatase activity in cellular membranes may be of

importance in the cellular transport mechanism. This has been claimed in the case of bile canaliculi,[29] the intercellular membranes of some renal cells,[22] and the secretory capillaries of the pancreas.[30]

Spargo[7] found no increase in acid phosphatase activity in the medulla of potassium-deficient rats, differing in this respect from Craig and Schwartz[9] and Pearse and Macpherson.[13] Spargo and Craig and Schwartz failed to detect the alterations in esterase activity observed by us and by Pearse and Macpherson. The latter investigators described a marked increase in TPN diaphorase and a reduction in DPN diaphorase activity in the medulla of potassium-deficient animals. We, on the other hand, were unable to confirm these observations. While various enzyme reactions were increased in the medulla, there was a uniform reduction of activity in the corticomedullary junction, localized to ascending limbs of Henle's loops. This was noted in preparations stained for adenosine triphosphatase, esterase, acid phosphatase, succinic dehydrogenase and DPN diaphorase. Decrease in activity was least noticeable with the stain for TPN diaphorase. Although conventionally stained sections did not show a significant dilatation of the ascending limbs of Henle's loops in our specimens, it has been pointed out[11] that a certain degree of obstruction of the lumens may occur as the result of proliferation of cells in collecting ducts. It is quite possible that this may lead to a mild internal hydronephrosis which in turn may account for the reduced enzymatic activity in the distal convoluted tubules and ascending limbs of Henle's loops. Such an internal hydronephrosis is apparently much more marked in weanling rats.[12]

A reduction of enzymatic reaction in atrophic tubules noted in occasional proximal convoluted tubules is typical of tubular atrophy resulting from a variety of causative factors.[1] In contrast to Pearse and Macpherson,[13] we were unable to detect an increase in esterase activity due to potassium deficiency in proximal convoluted tubules. On the basis of their histochemical observations, Pearse and Macpherson suggested that potassium deficiency affected protein metabolism, the permeability of tubules to protein, and also the processes of respiration and oxidative phosphorylation, particularly in tubules of the medulla and papilla. Whether changes in histochemical reactions permit such broad conclusions is open to some doubt.

It seems logical, however, to correlate these changes with the functional alterations that characterize potassium deficiency. It has been shown in the rat that potassium deficiency is accompanied by an inability to concentrate urine properly, or to produce a highly acid urine.[10,31] Although there are also alterations in cortical tubules, those

most specific for potassium deficiency are found in the medulla, as has been emphasized.[11]

It has been suggested by Hargitay and Kuhn[32] that the loops of Henle act as a countercurrent multiplier system for the concentration of urine. According to this concept, this mechanism would cause the interstitial tissue of the medulla to be hyperosmotic. This, in turn, would cause diffusion of water out of the collecting ducts with resulting concentration of the urine. Wirz, Hargitay and Kuhn,[33] on the basis of cryoscopic examination of slices made from concentrating rat kidneys, concluded that the osmotic pressure was identical for all tubular structures at a given level. They assumed that there was an increasing osmotic gradient from the cortex which was iso-osmotic with plasma to the tip of the papilla. Further support for the correctness of the "countercurrent multiplier system" hypothesis has been derived from the examination of fluid obtained from various portions of the nephron by Wirz,[34] and by Gottschalk and Mylle.[35] The latter gathered fluid from Henle's loops and adjacent collecting ducts in the papilla of the hamster's kidney. The fluid in both structures had a similar osmolality which was much higher than that of the blood plasma. In contrast, fluid from the cortical segments of the proximal convoluted tubules and the distal portions of the distal convoluted tubules was iso-osmotic and that of the distal convolutions hypo-osmotic to plasma.

In the kidney of the potassium-deficient animal, one is confronted with an obvious paradox. The concentrating power is severely impaired, but a number of enzymatic staining reactions in the two tubular segments of the nephron which are most responsible for urinary concentration are markedly increased. It is true enough that none of the enzymes demonstrated by histochemical methods are known to be implicated directly in the concentrating mechanism. It is obvious, however, that these enzymes participate in cellular metabolic processes, and an increase in their activity indicates increased cellular metabolism. In potassium deficiency, the hyperplasia of epithelium in the collecting ducts, particularly of intercalated cells, and the associated increase in enzymatic staining reactions in both the collecting ducts and the limbs of Henle's loops, could be interpreted as an expression of increased cellular activity aimed at overcoming the depressing effect of the deficiency on the normal function of these cells. Such an interpretation could also explain the increase in enzymatic activity in interstitial cells which, according to the theory of Hargitay and Kuhn,[32] participate in the concentrating mechanism. Indeed, Ullrich, Drenckhahn and Jarausch[36] have measured the osmotic pressure in the interstitial cells close to the tip of the papilla where the increase in histochemically

demonstrable phosphatase and esterase activity is most distinct, and found it practically identical with that of the urine.

Altogether it seems reasonable to assume that the changes found in the medulla in potassium deficiency of the rat are not a primary consequence of potassium depletion, but rather a nonspecific compensatory phenomenon aimed at overcoming the biochemical defect which occurs in renal cells deprived of this essential electrolyte.

Summary

Young albino rats of the Wistar strain were fed a complete synthetic diet deficient in potassium. Various enzymatic histochemical techniques were applied to the kidneys of such animals after they had received the experimental diet for 15 to 25 days. There occurred a striking increase in acid phosphatase and nonspecific esterase activity in the renal medulla, in the thin limbs of Henle's loops, in scattered interstitial cells, and, to a somewhat lesser degree, in collecting ducts. A distinct increase of adenosine triphosphatase activity was observed in the outer cell borders of collecting ducts. Stains for oxidative enzymes revealed less marked increase of activity in medullary structures.

There was an overall reduction in acid phosphatase, nonspecific esterase, adenosine triphosphatase, succinic dehydrogenase, DPN and TPN diaphorase activities in the ascending limbs of Henle's loops and distal convoluted tubules. The striking TPN diaphorase activity of the macula densa remained unchanged. Occasional atrophic tubules in the cortex revealed loss of staining by all techniques used.

The significance of the observations is discussed in relation to the severe functional alterations that occur in potassium deficiency, particularly the impairment of the kidney to concentrate urine properly. In the light of the "countercurrent multiplier system" theory of renal concentration, it is thought that the striking increase of enzyme activity in medullary cells implicated in this mechanism may indicate an attempt by these cells to overcome the depressing effect of potassium deficiency.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Counterstains were not used in any of the sections illustrated.

FIG. 1. Inner portion of the medulla in a rat after 14 days on the potassium-deficient diet. Paraffin section stained by Jones's periodic acid silver-methenamine technique. A positive reaction is given by the granules in the collecting ducts as well as by the cytoplasmic substance in the thin limbs of Henle's loops and interstitial cells. Basement membranes also stain strongly. × 700.

FIG. 2. Renal cortex of a rat after 25 days on the potassium-deficient diet. Formalin-fixed frozen section stained for TPN diaphorase. There is a marked reduction of enzymatic activity in atrophic proximal convoluted tubules. The cells of the macula densa on the upper left of the glomerulus show intense staining. × 450.

FIG. 3. Inner portion of the medulla of a normal control rat. Formalin-fixed frozen section stained for acid phosphatase. Fine granular deposits of lead sulfide indicating enzymatic activity are seen mainly in collecting ducts and to a lesser degree in the thin limbs of Henle's loops and interstitial cells. × 360.

FIG. 4. Inner portion of the medulla of a rat after 15 days on the potassium-deficient diet. Formalin-fixed frozen section stained for acid phosphatase. There is intense activity, most marked in the thin limbs of Henle's loops. Compare with Figure 3. × 360.

FIG. 5. Corticomedullary region in a normal rat kidney. Formalin-fixed frozen section stained for nonspecific esterase. The ascending limbs of Henle's loops show moderate staining in contrast to the much stronger staining of the proximal convoluted tubules shown at the upper edge of the photograph. × 90.

FIG. 6. Corticomedullary region of the kidney shown in Figure 4. Formalin-fixed frozen section stained for nonspecific esterase. Note the distinct diminution of enzymatic staining in the ascending limbs of Henle's loops as compared to the normal control section in Figure 5. × 90.

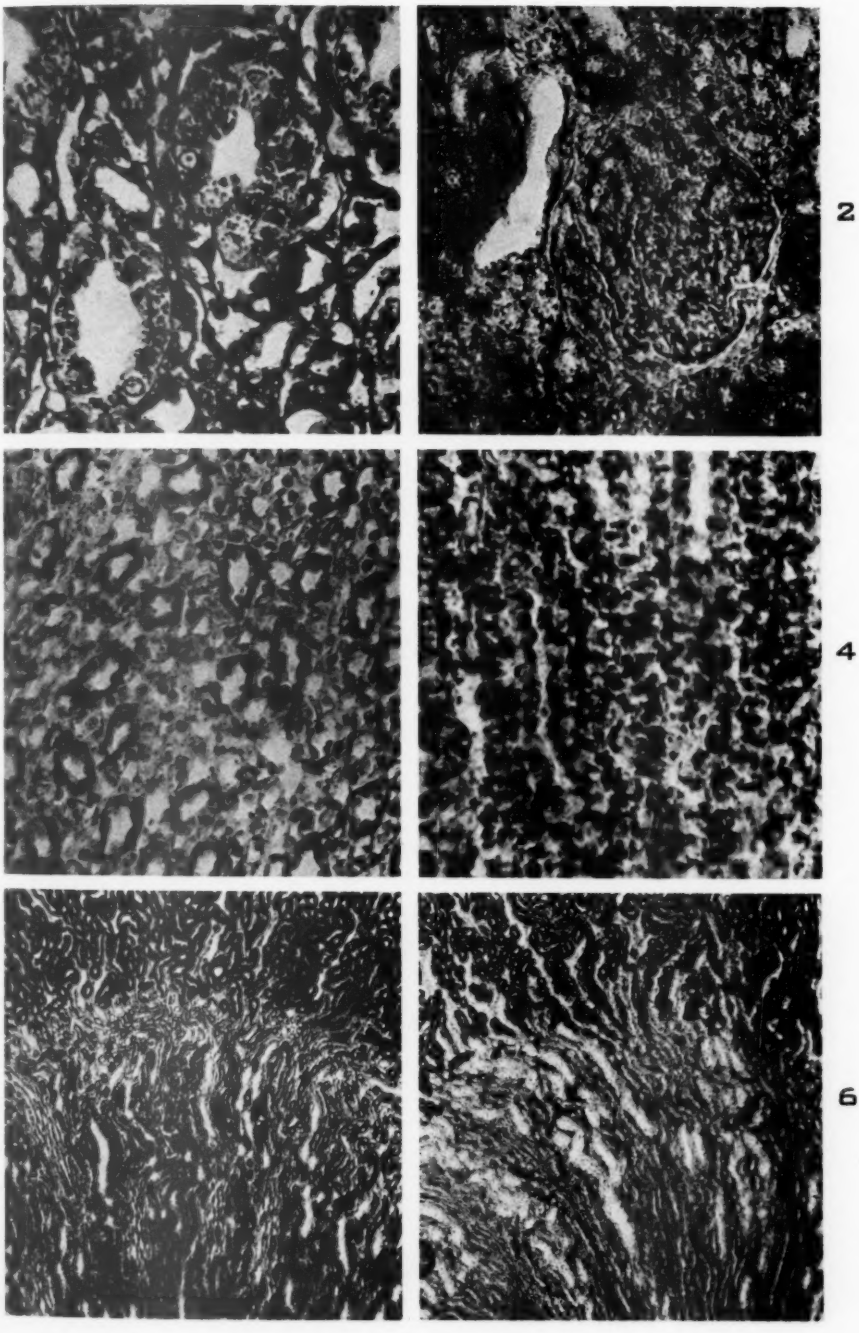
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FIG. 7. Inner renal medulla of a normal rat. Formalin-fixed frozen section stained for nonspecific esterase activity. The thin limbs of Henle's loops and occasional interstitial cells show more staining than the collecting ducts. × 360.

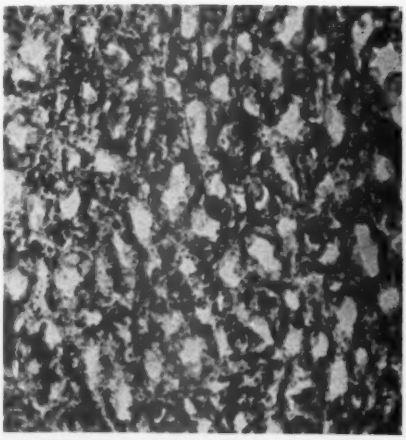
FIG. 8. An adjacent area in the kidney shown in Figures 4 and 6. Formalin-fixed frozen section stained for nonspecific esterase. There is striking staining of the thin limbs of Henle's loops and interstitial cells, and a somewhat less intense reaction in the collecting ducts. Compare with Figure 7. × 360.

FIG. 9. Inner medulla of a normal rat kidney. Formalin-fixed frozen section stained for adenosine triphosphatase activity after 5 minutes of incubation. Only the inner cell borders of collecting ducts are stained. Capillaries show almost no activity after this short incubation period. × 450.

FIG. 10. Inner medulla of the same kidney shown in Figures 4, 6 and 8. Formalin-fixed frozen section stained for adenosine triphosphatase activity after 5 minutes' incubation. There is markedly increased staining in the outer cell borders of collecting ducts. Compare with Figure 9. × 450.

FIG. 11. Outer portion of the renal medulla in a normal rat. Formalin-fixed frozen section stained for DPNH diaphorase activity. Staining in collecting ducts is most striking in some intercalated cells. The thin limbs of Henle's loops show weak activity. × 360.

FIG. 12. Outer portion of the medulla of the same kidney shown in Figures 4, 6, 8 and 10. Formalin-fixed frozen section stained for DPNH activity. There is a general increase in staining of cells composing the collecting ducts. There is also some increase in the staining intensity of the thin limbs of Henle's loops. Compare with Figure 11. × 300.



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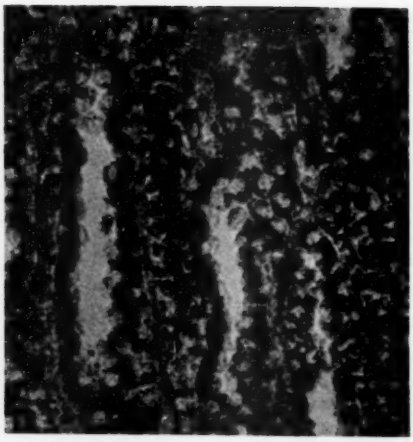

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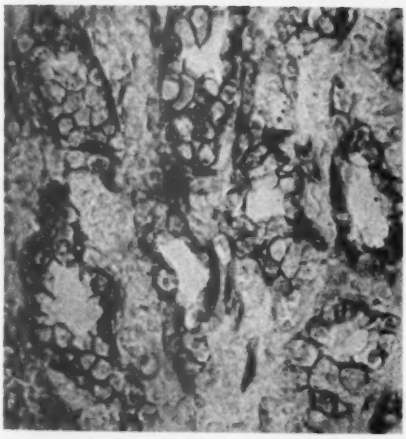

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FATAL ECLAMPTIC DISEASE OF PREGNANT RATS FED ANTI-VITAMIN E STRESS DIET *

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Diseases possibly analogous to human eclampsia and other fatal complications of human pregnancy are observed in a number of species of domestic or laboratory animals.[1,2] These maladies occur under such a wide range of experimental and naturally occurring conditions that any common etiologic factor is difficult to deduce, and none of the entities described has become widely accepted as an unquestioned counterpart of eclampsia as seen in the human patient.

Dietary factors have been implicated in a number of the pregnancy diseases reported in animals, just as they have been suspect in disorders of human pregnancy, but little exact knowledge of the roles of these factors has accrued. The known role of α-tocopherol as an antisterility factor in both sexes of several species of animals might be expected to provoke interest in the relation of the tocopherols to diseases of pregnancy. This has indeed been true, but the evidence thus far accumulated has not been sufficient to verify a decisive role for vitamin E in the prevention of the eclamptic disorders of human pregnancy.

Barrie, in 1939, reported "toxemia of pregnancy" in a small percentage of female rats given suboptimal amounts of vitamin E during gestation, and postulated a relationship to human toxemias of pregnancy.[3] Her work apparently was little noted, and the technique she described has not become recognized as an acceptable method of provoking complications of pregnancy.[4]

The present report offers detailed information concerning a fatal condition of pregnant rats receiving an anti-vitamin E stress diet. This regimen was employed in such fashion that gestation proceeded in a seemingly normal manner until the abrupt onset of symptoms leading to maternal fatality in the terminal phase of pregnancy. The disorder often culminated in an eclamptic episode and had clinical and pathologic aspects comparable to certain of the more acute maternal diseases of human pregnancy.

MATERIAL AND METHODS

Rats of Sprague-Dawley and Long-Evans strains were used initially. An inbred albino strain selected from these two parent stocks was later developed in order to minimize the pleuropneumonic disease prevalent in the original colony. The breeding colony and stock rats were fed a

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commercial (Rockland) ration as their sole diet. The young were ear-marked at weaning, and litter-mate controls were used when feasible. Young females were placed in breeding cages at about 75 days of age, at which time they ordinarily weighed approximately 200 gm. Matings were determined by vaginal smears, and the day on which spermatozoa were found was considered to be the first day of pregnancy.

The basic experimental diet contained 61 per cent corn starch, 20 per cent purified casein, 10 per cent brewer's yeast, 4 per cent HMW salt mixture,[5] and 5 per cent cod liver oil. These materials presumably contained very little tocopherol, and that present was assumed to be rendered ineffective by the oxidative action of the highly unsaturated fatty acids contained in the cod liver oil. The dietary ingredients were thoroughly mixed and allowed to remain at room temperature overnight. The food was then stored at −40 ° F. to minimize further oxidative changes. Male rats maintained on the diet became sterile and developed testicular atrophy after several weeks, but lived for several months in apparently good general health. The diet was begun on the morning of the 13th day of gestation when the pregnant state of the animal could be verified by palpation of the gravid uterus. Fresh supplies of the diet were removed from refrigeration daily and fed *ad libitum*. The rats were housed in metal cages with raised wire mesh floors; fresh tap water was supplied at all times.

The basic dietary regimen was modified in many ways; these included changes in all constituents of the diet, addition of vitamins and other supplements, and variations in storage methods.

Tissues for microscopic examination were fixed in 10 per cent formalin; sections were stained with hematoxylin and eosin and by other techniques as indicated.

Results

Clinicopathologic Observations

The pregnant rats ate the diet readily, gained weight at a normal rate, and usually exhibited no evidence of ill health until the 21st or 22nd day of gestation. At this time a majority of primigravid females succumbed in a rapidly progressive manner (Table I). Multigravid animals were somewhat less susceptible to the deleterious effects of the diet, but almost 50 per cent of those surviving the toxic diet during the first pregnancy succumbed at the second trial. A small number died during third and fourth pregnancies. Certain litters proved unusually resistant to the diet, but selection of breeders from these groups did not yield a consistently resistant strain. A very high percentage of maternal deaths occurred during the night in animals showing no sign of illness when observed on the previous evening. In a small propor-

tion of cases, events leading to death were observed. The animals first became restless, apprehensive, and showed ruffled coats and pallor of the conjunctivas, ears, and mucous membranes. Respirations became rapid, labored, and noisy; blood-tinged froth exuded from the nostrils.

TABLE I

Eclamptic Deaths in Pregnant Rats Beginning Anti-Vitamin E Stress Diet on the 13th Day of Gestation

Class	No. of rats	No. of deaths	Mortality (per cent)
I. First pregnancy	171	112	65.5
II. Second pregnancy:			
A. Given control diet in first pregnancy	18	9	50
B. Anti-vitamin E stress diet in first pregnancy (survivors of Class I)	59	27	45.8

Violent convulsions with erratic threshing movements and clonic contractions of the extremities usually ensued. After a few minutes a quiet, unresponsive state followed, and death quickly supervened.

A smaller number of animals displayed signs of distress, but survived the parturition period, usually with stillbirth of all or most of the young. Some of these mothers survived the puerperium and made an apparently complete recovery, while others became increasingly listless and died within a few days. Still others completed pregnancy in an entirely normal fashion.

When examined at necropsy, the animals dying abruptly presented characteristic lesions which included generalized visceral venous engorgement, cardiac dilatation, intense pulmonary congestion and hemorrhagic edema, and massive pleural effusion. Ascites of mild degree was seen occasionally, but peripheral edema was not evident. The kidneys were swollen and hyperemic. Focal or diffuse hemorrhages were frequently present in the kidneys and adrenals. Scanty amounts of hemoglobin-tinted urine occasionally were detected in the bladder and renal pelves. In a few instances the liver was swollen and displayed variable numbers of hemorrhagic foci. The brain was typically edematous and hyperemic. Intra-uterine bleeding was occasionally extensive, often minimal, and many times completely absent. No placental abnormalities were apparent. The fetuses were numerous, well developed, and usually alive and vigorous when examined immediately after maternal death.

The outstanding gross lesions found in animals dying after an illness of several days' duration were characterized by bilateral swelling, mottling, and discoloration of the kidneys, and varying degrees of adrenal alteration of similar nature. Visceral congestion, pulmonary

edema, and pleural effusion were usually not conspicuous in this group.

The pertinent microscopic features were chiefly those involving lungs, kidneys, adrenals, spleen, and liver.

Lungs. In addition to the abnormalities noted grossly, microscopic sections showed many fibrin thrombi in the smaller radicles of the pulmonary arterial system (Figs. 1 and 2). There was no apparent vasculitis, and the thrombi often appeared to lie free, with no attachment to vessel walls. Lungs of animals dying after a more protracted illness showed few specific lesions, but usually contained remnants of partially lysed or hyalinized thrombi (Fig. 3).

Kidneys. These typically showed occlusive lesions of glomerular vessels, accompanied by renal parenchymal abnormalities ranging from early tubular degeneration to complete bilateral cortical necrosis. Figure 4 illustrates the typical glomerular lesion; the arterioles and capillaries were occluded by homogeneous material with staining properties resembling those of agglutinated fibrin or platelets. This material appeared to be deposited within intact vessel walls. In some instances a narrow central lumen remained (Fig. 5) while other vessels appeared to be completely occluded. The kidneys of animals dying after an illness of several days' duration invariably displayed profound alteration. Most commonly this took the form of virtually complete bilateral renal cortical necrosis (Fig. 6). There were occlusive lesions of glomerular vessels similar to those in animals dying of more rapidly progressive disease, and also extensive thrombosis of interlobular and arcuate arteries. The occluding material in the larger vessels had the appearance of mixed thrombi formed by retrograde propagation following glomerular occlusion. Occasionally, when death occurred after several days' illness, extensive tubular necrosis was found with little or no demonstrable glomerular capillary occlusion (Fig. 7). The relatively rare type of renal lesion depicted in Figure 8 was found in animals dying after 7 or 8 days of illness. This lesion was characterized by extensive necrosis and dystrophic calcification of convoluted tubules; the glomeruli were ischemic but otherwise normal. The tubular alteration was similar to that described by Barrie[8] and might be considered analogous to certain of the tubular nephropathies of the human species (Fig. 7).

Adrenals. The adrenals were frequently the site of focal or massive cortical necrosis and hemorrhage. In some instances, thrombi could be identified within the cortical sinusoids and arterioles in and adjacent to the areas of necrosis (Fig. 9).

Spleen. The spleens of animals dying abruptly presented a characteristic microscopic appearance; the pulp was typically engorged, and

many reticuloendothelial cells were seen to be greatly distended with disintegrating erythrocytes (Fig. 10). This correlated with the observation of hemoglobinuria in those animals in which urine was observed in the bladder or renal pelves. The sudden development of this disturbance was indicated by the absence of abnormal amounts of hemosiderin in the reticuloendothelial system, and by the lack of demonstrable hemolysis in most blood samples drawn shortly before the onset of symptoms.

Liver. No characteristic hepatic lesions were found in most instances.[6] Occasionally a liver exhibited scattered periportal foci of cellular degeneration or necrosis, thrombosis, and hemorrhage. In 4 instances in over 300 deaths, very extensive hepatic alteration of this type occurred (Fig. 11). Formation of large rounded intracytoplasmic bodies within liver cells was the earliest change detected, and often was found without accompanying hemorrhage or thrombosis. The bodies appeared as colloid droplets with eosin stains, and were strongly positive to the periodic acid-Schiff stain (Fig. 12). In more advanced lesions many of the liver cells containing such droplets showed various stages of disintegration and necrosis, and the livers exhibited extensive thrombosis and hemorrhage in periportal zones (Fig. 13).

Other Organs. No abnormalities except hyperemia and edema were found in the brains of the affected animals. No characteristic lesions of the uterus, placenta, or fetus were detected. The uterine veins were conspicuously patent and filled with fluid blood when examined soon after death of the animals. This was in contrast to the thrombosis found elsewhere.

Animals sacrificed on the 21st day of gestation, before symptoms of illness had begun, showed no evidence of abnormality. This was true also of animals which completed pregnancy uneventfully, as well as those which displayed signs of distress followed by clinical recovery.

Effects of Modification of Diet

Lipids. The lipid content was found to be a critical component in the basic diet (Table II). Fresh cod liver oil at the 5 per cent level was relatively innocuous, but toxicity was greatly increased with larger oil content or partial peroxidation. Substitution of fresh lipids such as butter or corn oil eliminated the toxic quality of the diet, but use of partially oxidized lard, butter, or corn oil resulted in highly toxic diets, as did linoleic acid preparations. The original supplies of cod liver oil, lard, and corn oil had been stored at 5° C. for several years and had undergone oxidative changes very slowly. Additional supplies of cod liver oil were partially rancidified much more rapidly by heating and

aerating on a steam bath to attain the desired peroxide number. Similar results were produced by mixing the oil or fat with the other dietary ingredients and allowing the mixture to stand at room temperature until the desired peroxide level was attained. This was done with the

TABLE II

The Effect of Peroxidation of Lipid Constituents of Anti-Vitamin E Stress Diets on Mortality Rates of Pregnant Rats

Lipid used	Per cent in diet	Peroxide number*	No. of rats	No. of deaths	Mortality (per cent)
Cod liver oil					
A. Fresh	5	16	6	1	17
B. Fresh	15	16	18	9	50
C. Heated and aerated	5	108	6	5	83
D. Heated and aerated	5	319	12	3	25
E. Diet aged at room temperature, 21 mos.	5	10	5	2	40
F. Diet E, acetone-extracted	0	0	8	2	25
G. Acetone extract of Diet E used to reconstitute diet	5	10	7	0	0
Corn oil					
A. Fresh	5	0	6	0	0
B. Aged at 5° C.	10	291	4	3	75
Lard, aged at 5° C.	10	344	10	8	80
Butter					
A. Fresh	10	0	5	0	0
B. Aged 60 days in diet at room temperature	10	100	5	3	60
Linoleic acid					
A. Fresh	10	12	7	3	43
B. Oxidized at room temperature	10	1630	5	1	20
C. Diet B, diluted with control diet ingredients	5	1630	9	7	78
Methyl linoleate					
A. Oxidized at room temperature	10	304	5	3	60
B. Diet kept at room temperature 12 additional days	10	1545	4	0	0
Oleic acid					
A. Fresh	10	0	5	0	0
B. Diet kept at room temperature 135 days	10	0	5	0	0

* Peroxide values are expressed as mEq. of peroxide per kg. of lipid.

diets containing butter, linoleic acid, or methyl linoleate. The diets could be best maintained at a desired level of peroxide content by using an oil or fat of known peroxide value and storing the mixed diet in a refrigerator at −40° F.

It was noted that several diets with very high peroxide values appeared to be relatively nontoxic. However, the animals ate these diets very poorly, became rather emaciated, and produced inferior litters containing a high proportion of stillborn and underdeveloped

young. When these diets were diluted with equal parts of control diet minus lipid, they were eaten readily, and there was a high maternal mortality rate, as shown in the case of linoleic acid (Table II). This example illustrates the general observation that in order to produce the syndrome of eclampsia consistently, it was necessary to provide a diet which was eaten readily and maintained an apparently normal state of health in the pregnant female until the terminal phase of gestation. Cod liver oil diets exposed to air at room temperature rather quickly acquired a high peroxide level, which then gradually subsided to a value approaching zero. These "aged" diets still produced some toxic effect even after thorough extraction with acetone to remove lipids and lipid peroxides. The extracted oil, on the contrary, appeared to be nontoxic when added to fresh dietary ingredients.

TABLE III

The Effect of Modification of Non-lipid Constituents of Anti-Vitamin E Stress Diet on Mortality Rates of Pregnant Rats

Modification of diet	No. of rats	No. of deaths	Mortality (per cent)
Proteins replacing casein			
Alpha protein (soybean), 20%	4	4	100
Gelatin, 20%	5	0	0
Gelatin, 10%; casein, 10%	3	3	100
Gluten, 20%	3	2	67
Lactalbumin, 20%	3	2	67
Zein, 20%	3	2	67
Brewer's yeast omitted	35	6	17
Plus B vitamin mix	13	5	38
Plus liver powder, 10%	6	4	67
Vitamin supplements			
Ascorbic acid, 1%	4	3	75
Vitamin supplement, 2.2% (vitamin E omitted)	17	4	23.5
Antioxidants			
Ditertiary butyl-p cresol, 0.25%	4	2	50
Nordihydroguaiaretic acid, 0.5%	4	1	25
DPPD, 0.05%			
(a) Days 13 to 22	12	0	0
(b) Days 1 to 12	8	5	63
Choline chloride, 1%	4	2	50
Salt mix omitted	7	4	57

Carbohydrates. Modifications of the basic diet, with corn starch, dextrose, sucrose, polished rice, or a combination of several of these ingredients as the source of carbohydrate, showed similar degrees of toxicity in the pregnant rat.

Minerals. The basic diet remained quite toxic when the mineral

supplement was omitted (Table III). Addition of sodium chloride to the basic diet in amounts as high as 4 per cent of the diet caused no apparent variation in the effects produced.

Protein. The basic diet remained quite toxic when a number of other protein preparations replaced casein at the 20 per cent level (Table III). Gelatin was the only protein tested that seemed protective against eclampsia. The diet containing 20 per cent gelatin, however, was eaten very poorly; the pregnant animals gained little or no weight, and the young were small and weak at birth, with a high incidence of stillbirth and early neonatal death. A diet containing 10 per cent gelatin and 10 per cent casein was eaten readily, maternal weight gains were in the low normal range, and fatal eclampsia occurred in each of 3 subjects.

Brewer's Yeast. Omission of this ingredient from the experimental diet gave variable results, but in general the maternal mortality rate was rather low. The diet without yeast was observed to oxidize readily and attain high peroxide levels; it was eaten rather poorly. Addition of acetone-extracted pork liver to the yeast-free diet gave results comparable to the basic diet. Addition of a B-vitamin mix to give a concentration of *p*-aminobenzoic acid, 10 gm.; niacin, 9 gm.; riboflavin, 2 gm.; pyridoxine hydrochloride, 2 gm.; thiamine hydrochloride, 2 gm.; calcium pantothenate, 6 gm.; biotin, 40 mg.; folic acid, 180 mg.; and vitamin B-12, 2.7 mg. per 100 pounds of diet also resulted in an appreciable increase in the apparent toxicity of the yeast-free diet, although not to the level of the basic diet.

Vitamin Supplements. Addition of a commercial mixture (National Biochemicals Company) designed to supply optimum amounts of all known vitamins except vitamin E considerably reduced maternal mortality rates. This diet was eaten readily. Pregnant animals made normal weight gains, and most of those which did not succumb to eclampsia produced healthy litters. Ascorbic acid at high levels was ineffective. In view of the toxicity of fresh cod liver oil at the 15 per cent level, it appeared unlikely that vitamins A or D possessed any protective property. This assumption applied to the B-vitamin group, as indicated by the results obtained with brewer's yeast or B-vitamin supplements of yeast-free diets.

Vitamin E. Supplementation of the diet by tocopherol preparations was the only completely successful means found to prevent death of the pregnant rat receiving the basic eclamptogenic diet. The results summarized in Tables IV and V show the effectiveness of several different tocopherol preparations given in a variety of ways. DL-α-tocopheryl acetate was effective when given prophylactically as an addition to the

TABLE IV

The Effect of Tocopherol Preparations in Prevention of Eclamptic Deaths of Pregnant Rats Given Anti-Vitamin E Stress Diets

Vitamin E preparation	Dosage	Route of administration	Days of gestation given	No. of rats	No. of deaths	Mortality (per cent)
α-Tocopheryl acetate	0.5%	In diet	1-12	3	0	0
α-Tocopheryl acetate	0.05%	In diet	1-12	4	0	0
α-Tocopheryl acetate	0.2%	In diet	13-22	4	0	0
α-Tocopheryl acetate	80 mg.	Oral	19-21	2	0	0
α-Tocopheryl acetate	80 mg.	Oral	19-22	5	0	0
Tocopherol phosphate	50 mg.	Subcutaneous	19th	3	0	0
Tocopherol phosphate	50 mg.	Subcutaneous	20th	4	2	50
Tocopherol PGS*	100 mg.	Subcutaneous	19th	2	0	0
Tocopherol PGS	100 mg.	Subcutaneous	20th	8	0	0
Tocopherol PGS	25 mg.	Subcutaneous	19th	15	0	0
Tocopherol PGS	25 mg.	Subcutaneous	20th	10	1	10
Tocopherol PGS	25 mg.	Subcutaneous	21st	8	5	62.5
Tocopherol PGS	25 mg.	Intravenous	21st (evening)	11	0	0
Lettuce	ad lib.	With diet	1-12	9	2	22
Lettuce	ad lib.	With diet	13-22	10	0	0
Carrots	ad lib.	With diet	13-22	4	3	75

TABLE V

Protective Effect of Past Tocopherol Administration in Rats Subjected to Anti-Vitamin E Stress Diet During Second Pregnancy

Treatment during first pregnancy		Route of administration	No. of rats	No. of deaths	Mortality (per cent)
Preparation	Dosage				
Tocopherol PGS*	100 mg.	Subcutaneous	11	0	0
Tocopherol PGS	25 mg.	Subcutaneous	19	4	21
Tocopherol Phosphate	50 mg.	Subcutaneous	1	0	0
α-Tocopheryl acetate	240 mg.	Oral	3	0	0
α-Tocopheryl acetate	0.04%	In diet, days 1-12	1	1	100
α-Tocopheryl acetate	0.2%	In diet, days 13-22	3	0	0
α-Tocopheryl acetate	0.5%	In diet, days 1-12	3	0	0
Lettuce	ad lib.	With diet, days 1-12	5	2	40
Lettuce	ad lib.	With diet, days 13-22	8	5	62.5

* See text.

stock diet during the first 12 days of pregnancy, when given mixed with the toxic diet, or as an oral supplement late in pregnancy. Fresh lettuce, fed *ad libitum,* was also quite effective when given with the anti-vitamin E stress diet, but was not completely protective when given prophylactically during early gestation, probably because the amount of lettuce consumed resulted in relatively little increase in stored tocopherol. Carrots were eaten very readily, but, in contrast to lettuce, provided no protection against eclampsia when fed as a supplement to the basic diet.

Tocopherol was also quite effective when given in water-soluble form by parenteral injection, particularly as D-α-tocopheryl Polyethylene Glycol 1000 Succinate (Tocopherol PGS, supplied by Dr. P. L. Harris, Distillation Products Industries, Rochester, New York). The phosphoric acid ester-disodium salt of DL-α-tocopherol (tocopherol phosphate) also appeared to be effective in limited trials. These preparations were not always protective, however, if given late in pregnancy (Table IV), and in no instance was treatment with any tocopherol preparation effective if given after the onset of symptoms. Intravenous administration of Tocopherol PGS was quite successful, however, when given as late as the evening of the 21st gestational day. This was in sharp contrast to subcutaneous administration of the same drug, which was quite ineffective on the 21st day, although completely protective on the 19th day, and almost completely protective on the 20th day.

The protective effect of previous administration of tocopherol is illustrated in Table V, in which maternal deaths associated with a second anti-vitamin E stress pregnancy are tabulated. These animals all survived their first pregnancy when subjected to the eclamptogenic dietary regimen supplemented by vitamin E. No eclamptic deaths resulted in the 11 animals which had received 100 mg. of Tocopherol PGS (about 25 mg. of tocopherol) during the first pregnancy, whereas 4 of 19 died in the group which had 25 mg. of the drug. This was still considerably lower than the 50 per cent death rate shown in the second pregnancy control group in Table I. In contrast, the group given lettuce during the first pregnancy had a 54 per cent mortality, which was not appreciably different from the control group.

Antioxidants. Several antioxidants, at relatively high levels, protected against maternal eclampsia partially (Table III), although they apparently were rather toxic and resulted in poor litters in the survivors. N, N′-diphenyl-p-phenylenediamine (DPPD) was by far the most effective, although toxic at higher levels. At the .05 per cent level it was relatively nontoxic and gave complete protection against

eclampsia when added to the eclamptogenic diet beginning on the 13th day of pregnancy. It was quite ineffective at this level, however, when given during the first part of pregnancy.

Discussion

The data clearly indicate a direct relationship between the development of an acute fatal disease of pregnant rats and a dietary regimen featuring substantial amounts of partially oxidized polyunsaturated fatty acids. This diet is adjudged to be virtually devoid of vitamin E, and because of its pro-oxidative properties, is thought to cause rapid depletion of body stores of tocopherol. The protective action of α-tocopherol is presumably a function of the antioxidant nature of this compound, although a possible specific role in relation to an enzyme system or other biochemical entity cannot be excluded from consideration. The observation that certain synthetic antioxidants, notably DPPD, can partially take the place of α-tocopherol in the prevention of this eclamptic disorder of pregnancy is in accord with observations demonstrating the ability of these compounds to delay or prevent the appearance of such manifestations of vitamin E deficiency as muscular dystrophy, liver necrosis, and sterility. It has not yet been established whether the antioxidants act by sparing tocopherols in the diet or body tissues, or by actually substituting for absent tocopherols.[7] The present study does not serve to elucidate this matter. The partial protection accorded by polyvitamin supplements may likewise be due to the antioxidant activity of some component, since these substances were observed to retard peroxide formation in diets exposed to air at room temperature.

The lethal effects of diets containing polyunsaturated fatty acids apparently are largely dependent upon oxidative reactions, but may not be caused directly by peroxides, as indicated by the results obtained with aged acetone-extracted diets. The fatal disorder produced by such peroxide-free diets may be due to the lipoprotein copolymers formed as end products of oxidation of fatty acids in the presence of proteins.[8] Alternatively, it may be that prolonged oxidation results in complete elimination of vitamin E from the diet. The latter thus becomes capable of causing fatal disease in animals with unusually low tocopherol reserves. It is noteworthy, at any event, that the diet prepared with oleic acid, which did not form peroxides, exhibited no toxicity either in the fresh state or after prolonged exposure to air at room temperature.

The evidence at hand strongly indicates that the pathogenetic process involved in the eclamptic disease of pregnant rats is the sudden de-

velopment of thrombosis and fibrin thrombo-embolism. Preliminary investigation of blood coagulation components has failed to disclose the nature of this apparent hypercoagulability. The absence of demonstrable abnormality in the uterus and its contents in most instances eliminated this site as an obvious source of excess thromboplastin. When massive intra-uterine hemorrhage was found, it could have been secondary to depletion of fibrinogen and other components of the coagulation complex consequent to the initial thrombosing disorder. Vitamin E has been acclaimed as an anticoagulant effective in preventing thrombo-embolism in human subjects by increasing antithrombic activity of plasma. This role has been questioned,[9] however, and no alteration of antithrombic activity has been discerned in vitamin E depleted rats, eclamptic or otherwise. Thrombocytopenia and hemolysis have been observed terminally in several eclamptic animals. Massive agglutination and disintegration of platelets have been thought to account for certain categories of intravascular thrombosis in the human species, as in thrombocytopenic thrombotic purpura.[10] This sequence of events has not been shown to occur in the present instance, however, and a rapid fall in the platelet count might be expected to occur in response to any state of disseminated intravascular thrombosis.

It has been reported recently that erythrocyte stroma constitutes a rich source of thromboplastic substance (erythrocitin),[11] and clinical states of intravascular thrombosis associated with hemolysis are widely recognized.[12] Vitamin E deficiency is known to enhance the susceptibility of erythrocytes to hemolysis.[13] One thus may postulate hypercoagulability and intravascular thrombosis on the basis of abnormal thromboplastic activity due to release of erythrocytin from erythrocytes hemolyzed as the result of depletion of vitamin E. Proof of this supposition, however, is as yet entirely lacking since no evidence of abnormal thromboplastic activity of plasma has been detected. That the thrombotic tendencies may be due to alterations of vessel walls rather than of the coagulation system is suggested by the recent work of Golberg, Smith and Martin.[14] These authors found iron deposition and thrombotic occlusion of renal glomerular capillaries in rabbits subjected to overloading by means of prolonged parenteral administration of colloidal iron. Later work by these authors emphasized the similarity of lesions caused by iron overload to those of vitamin E deficiency.[15]

Any comparison of the lesions in this condition of rats with disorders of human pregnancy must take into consideration the quantitative differences in gestation in the two species. It is reasonable to

assume that a fatal disorder developing during a gestation of 22 days could have a much more abbreviated time span than one associated with a gestation of 280 days. That this is probably true is attested by the lack of obvious abnormality in the potentially eclamptic rat until a few hours before death, plus the effectiveness of therapy until this final time interval is reached. With these temporal considerations in mind, certain analogies may be drawn. The hepatic lesions in the two species appear to be remarkably similar, if not identical, although the comparison is weakened by the much lower incidence of this lesion in the rat. The similarity in the two species of terminal renal failure due to massive bilateral renal cortical necrosis is also striking. These cases, as well as many of those with hemorrhage and circulatory collapse, are quite reminiscent of *abruptio placentae,* placental infarction, and retained dead fetus with fibrin embolism classified as "thromboplastin complications of pregnancy"[16] in human subjects.

The pathogenetic interpretation of the renal lesions of human eclampsia is hampered by the curiously disparate conclusions reached by those who have investigated the pathologic features of that disease. A dichotomous body of opinion came into being with the early studies made in the late 19th century, and this still persists today. Schmorl, among the early students of eclampsia, described lesions in the kidneys, adrenals, liver, and lungs associated with intravascular thrombosis of these organs.[17,18] His views received support, notably from Lubarsch,[19] but later Fahr expressed a differing view that the essential disorder was a glomerulonephritis of membranous type, with lesions very similar to those seen in lipoid nephrosis.[20] Recent publications have tended to emphasize the renal alterations as the basic lesions of eclampsia, and have modified or amplified Fahr's interpretation of these as being predominantly concerned with alterations of the glomerular vessel walls or intercapillary substance (mesangium).[21-24] The authors of these publications either ignore Schmorl's early views stressing the significance of intravascular thrombosis or dismiss them as being unsubstantiated by later work. In contrast to this viewpoint, McKay and co-workers[25] described prominent thrombotic lesions in various organs of all of their reported cases of eclampsia. Their descriptions were strongly reminiscent of Schmorl's, and they interpreted eclampsia as being primarily a disease of intravascular thrombosis, probably associated with vasospasm as in the generalized Shwartzman reaction.[26] The report by Govan[27] is unusual in that he discussed extensive changes in vessel walls and "mesangium," and in addition described occlusion of glomerular capillary loops by hyaline thrombi in all of 33 reported cases. This might be interpreted as a reconciliation

and synthesis of the two opposing views long expressed regarding the nature of glomerular lesions of eclampsia. It is of interest that a similar interpretation might be made in regard to the renal glomerular lesions in the eclamptic disease of the rat. The apparent differences in the two species might then be viewed as quantitative ones, correlated with differences of duration of the disease process. The more conspicuous alterations of vessel walls and intercapillary substances commonly described in the human lesion might characterize a more prolonged state of disease, and the prominence of thrombosis as usually seen in the rat might exemplify a fulminant disorder of short duration.

Summary

A high percentage of female rats died during the terminal phases of pregnancy when given a diet deficient in vitamin E and containing substantial amounts of partially peroxidized polyunsaturated fatty acids beginning on the 13th day of gestation. The other constituents of the diet may be varied greatly, but diets that were eaten poorly and provided obviously poor fetal and maternal nutrition were relatively ineffective in causing maternal death. Maternal death was often preceded by eclamptic convulsions and was associated with widespread intravascular thrombosis, affecting especially the lungs and kidneys. Preliminary investigations of the coagulation mechanism have not provided a pathogenetic explanation for intravascular thrombosis. The disease in the rat has clinical and pathologic points of similarity to eclampsia and certain other fatal maternal disorders of human pregnancy. The animals were effectively protected against the development of this disease state by the administration of α-tocopherol in adequate amounts. Partial protection was also provided by antioxidants and by polyvitamin supplements.

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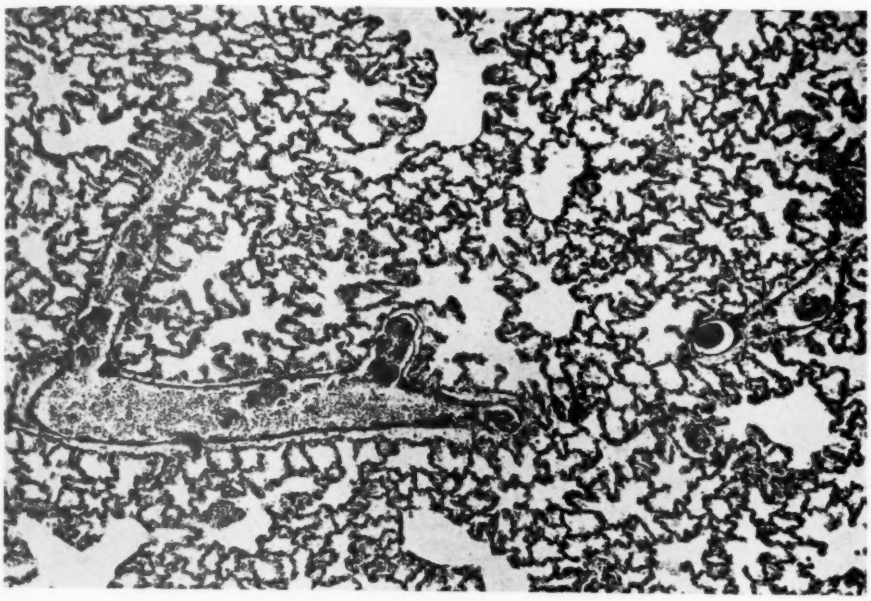
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Lung of eclamptic rat, fed basic anti-vitamin E stress diet minus brewer's yeast. Death at time of parturition. There are numerous fibrin thrombi in smaller branches of pulmonary arteries. Intense engorgement of alveolar capillaries is accompanied by little edema. Allochrome stain. × 100.

FIG. 2. Lung of eclamptic rat, fed basic anti-vitamin E stress diet. Intrapartum death, 22nd day of gestation. Intense pulmonary congestion and edema, with thrombus in medium-sized artery. Longitudinal orientation of fibrin strands is seen. Allochrome stain. × 150.

FIG. 3. Lung of eclamptic rat, fed basic anti-vitamin E diet plus 10 per cent cellulose. Postpartum death, 22nd day of gestation, after 24-hour illness. Pulmonary congestion and edema are not conspicuous. The fragments of hyaline material attached to the vessel wall and surrounded by leukocytes are typical of partially lysed thrombi found in animals dying delayed deaths. Hematoxylin and eosin stain. × 300.



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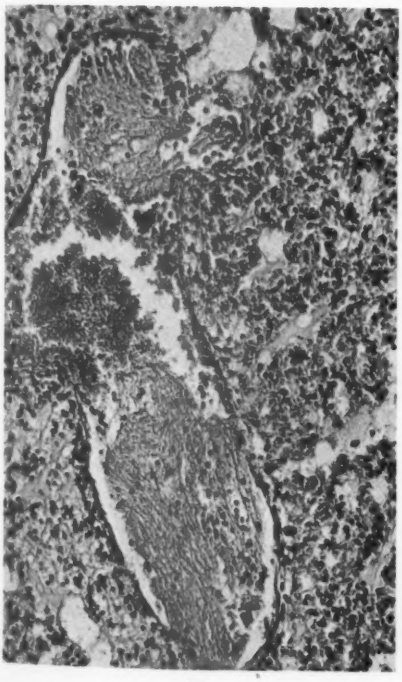

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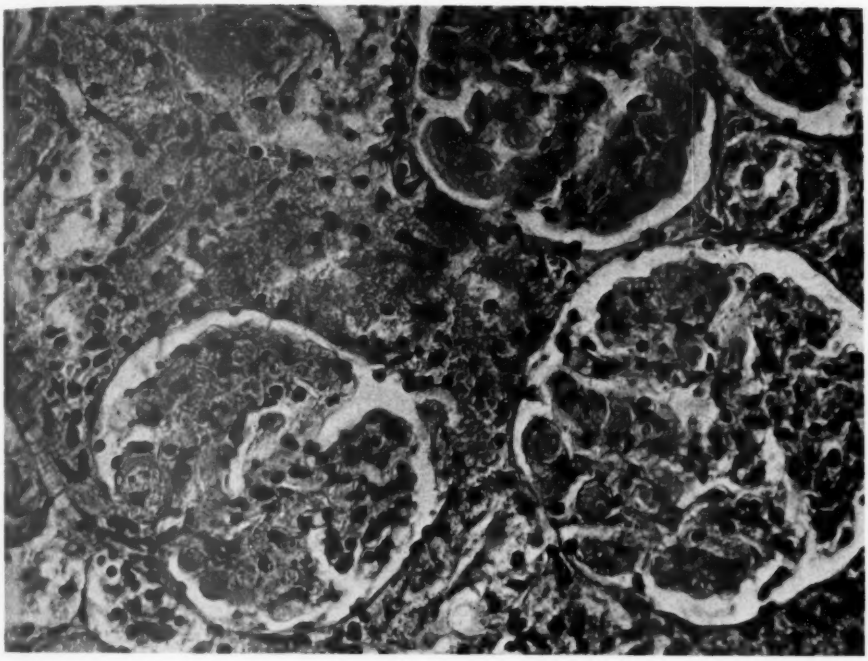
FIG. 4. Kidney of eclamptic rat, fed basic anti-vitamin E stress diet. Death, third postpartum day, after several days' illness. There is extensive occlusion of glomerular capillaries and arterioles by hyaline thrombi. Tubules show rather advanced degenerative changes. Hematoxylin and eosin stain. × 300.

FIG. 5. Kidney of eclamptic rat, fed basic anti-vitamin E stress diet. Intrapartum death. Partial occlusion of vessels by hyaline material applied to walls of glomerular arterioles and capillaries. Allochrome stain. × 300.



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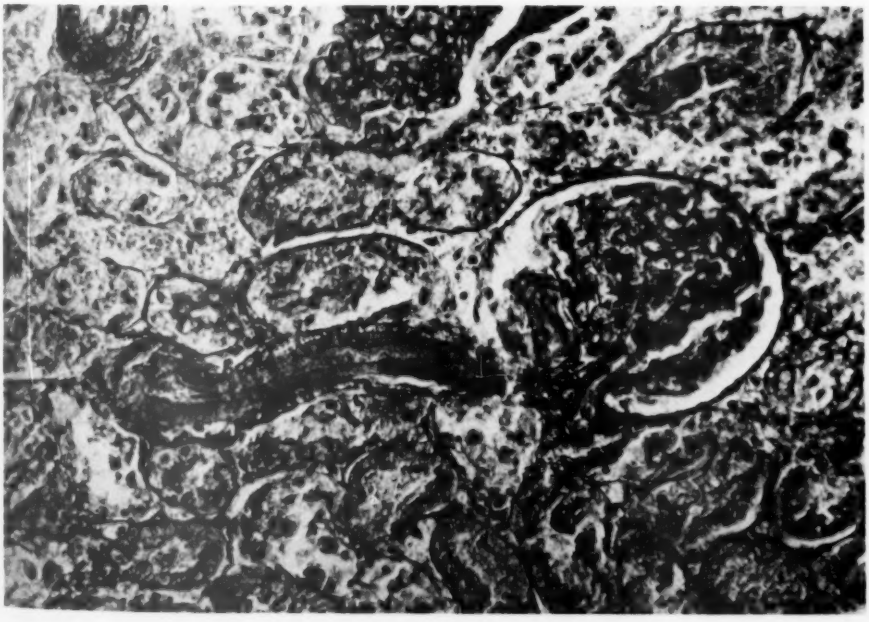

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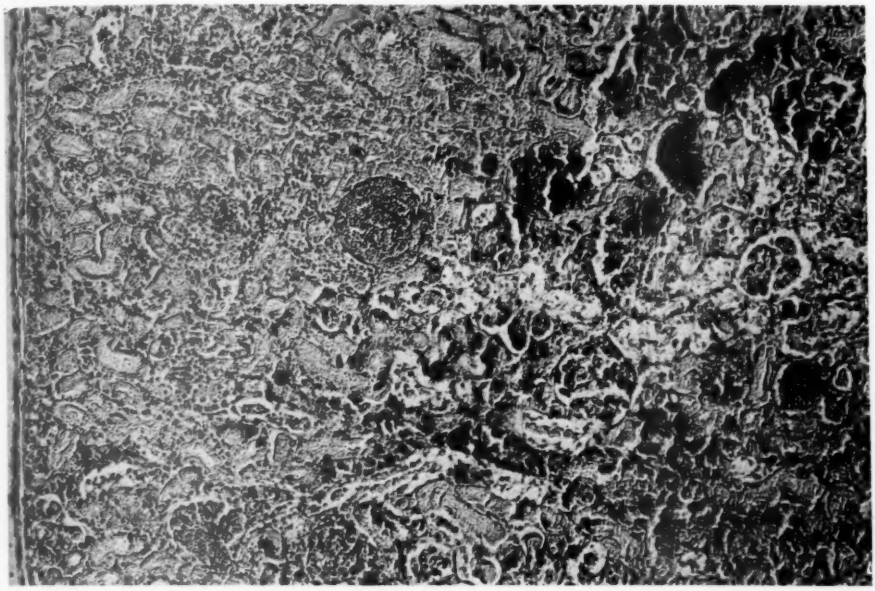
FIG. 6. Massive bilateral renal cortical necrosis; eclamptic rat fed basic anti-vitamin E stress diet with 10 per cent rancid corn oil replacing cod liver oil. Death, third day postpartum. Litter stillborn. There is thrombosis of glomerular vessels and arcuate and interlobular arteries. A viable glomerulus is seen in the juxtamedullary zone. Hematoxylin and eosin stain. × 100.

FIG. 7. Renal tubular necrosis; eclamptic rat fed basic anti-vitamin E stress diet. Postpartum death, one week after birth of litter. Only two of young were live-born. Glomeruli are viable but ischemic in appearance, with mild thickening and fibrillary changes of capillary walls. Hematoxylin and eosin stain. × 300.

FIG. 8. Renal tubular necrosis and dystrophic calcification; eclamptic rat fed basic anti-vitamin E stress diet plus 4 per cent added sodium chloride. Death, one week following parturition. Live-born litter of 7 discarded after 48 hours. Glomeruli appear contracted and ischemic, but are otherwise normal. Extensive calcification of tubular debris has caused disruption of tissue in sectioning. Hematoxylin and eosin stain. × 175.



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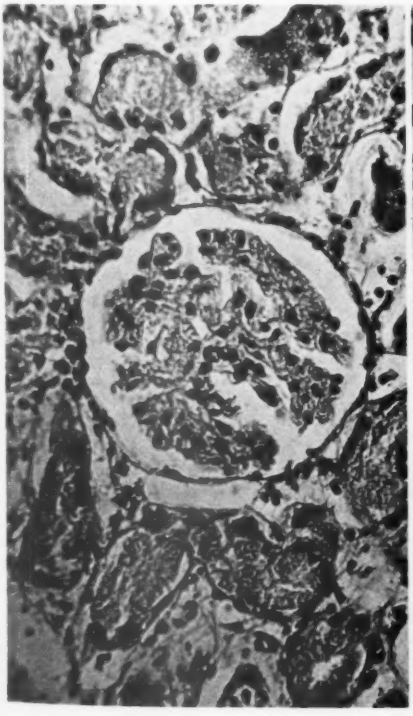

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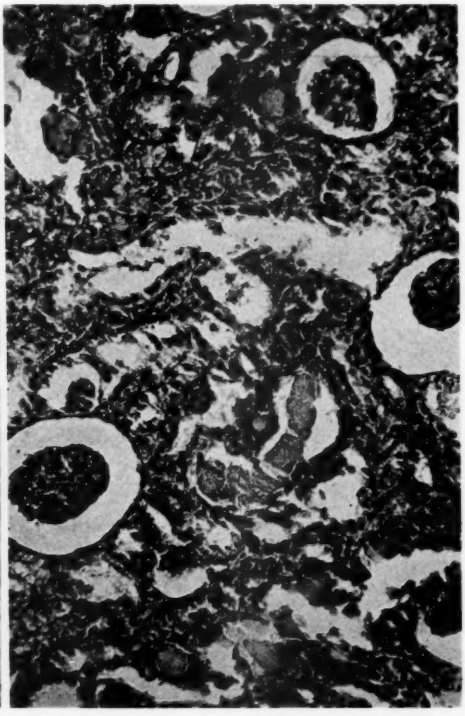

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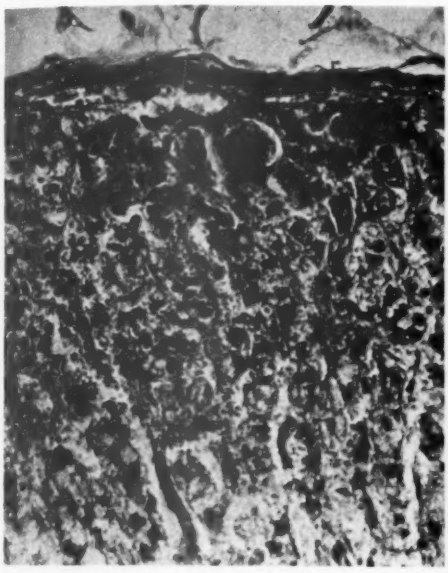
FIG. 9. Adrenal, showing cortical necrosis, with thrombosis of arterioles and sinusoids, from same case as Figure 2. Hematoxylin and eosin stain. × 300.

FIG. 10. Spleen of eclamptic rat fed basic anti-vitamin E stress diet. Intrapartum death, 22nd day of gestation. Splenic pulp contains many macrophages engorged with disintegrating erythrocytes. Hematoxylin and eosin stain. × 300.

FIG. 11. Liver of eclamptic rat fed basic anti-vitamin E stress diet, showing extensive peripheral lobular necrosis, thrombosis, and hemorrhage. Found dead, morning of 22nd day of gestation. Lungs and kidneys showed moderate degree of intravascular thrombosis. Uterus contained 16 dead fetuses of normal appearance, and there was no uterine hemorrhage. Hematoxylin and eosin stain. × 175.



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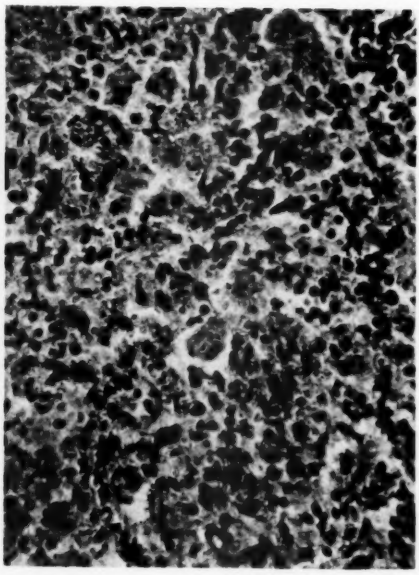

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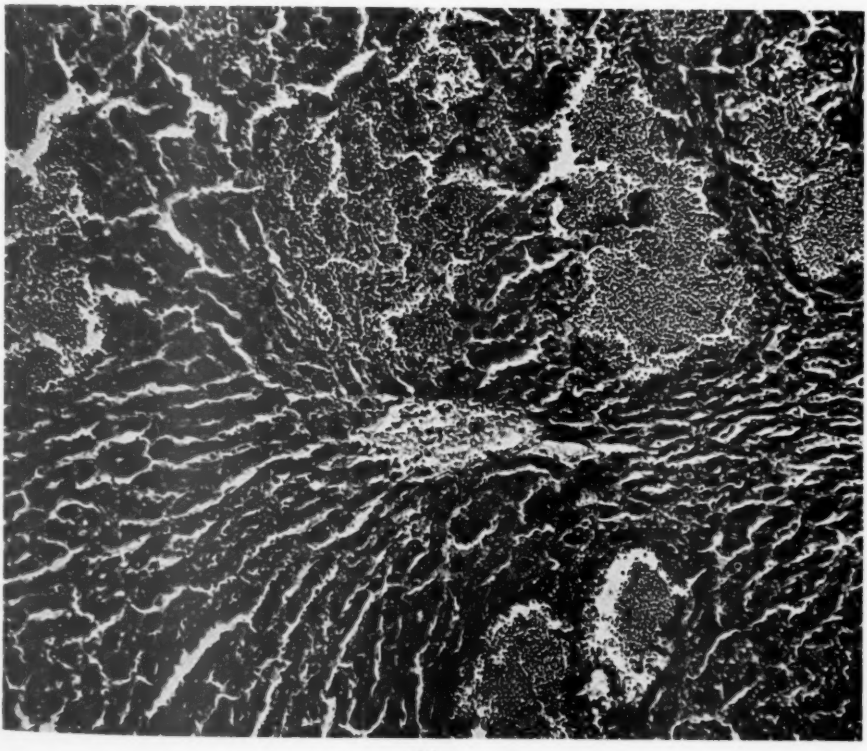

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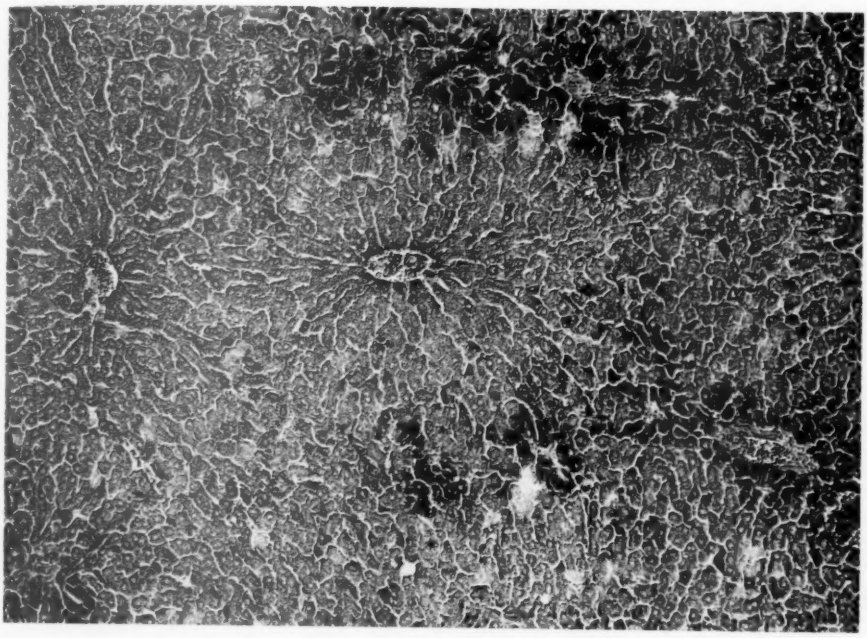
FIG. 12. Liver of eclamptic rat fed basic anti-vitamin E stress diet. Death, 24 hours following stillbirth of 10 young. The low-power view shows periportal localization of lesions characterized by "colloid droplets" in liver parenchymal cells, with beginning necrosis of liver cells and thrombosis of sinusoids. Extensive thrombotic lesions of kidneys and adrenals were present with minimal pulmonary involvement. Periodic acid-Schiff stain, no counterstain. × 100.

FIG. 13. Liver, higher magnification of periportal zone from Figure 12. The PAS-positive intracytoplasmic bodies are evident. In areas of liver cell disruption, these rounded masses are seen embedded in thrombi within sinusoids. PAS stain, no counterstain. × 300.



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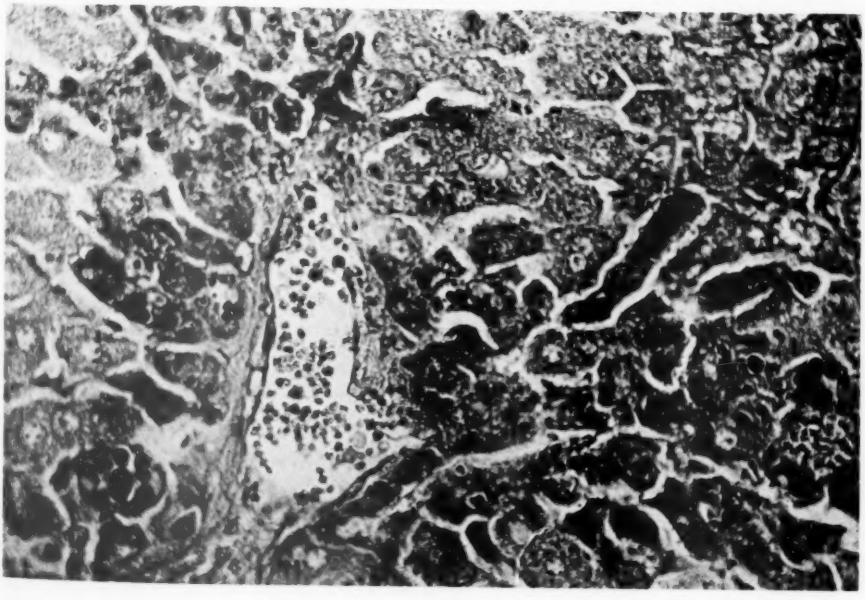

13

RADIATION-INDUCED GASTROINTESTINAL DEATH IN THE MONKEY *

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This report describes the unique changes produced in the gastrointestinal tract of *Macaca mulatta* monkeys by supralethal doses of gamma radiation, and discusses some of the inferences which may be made concerning the pathogenesis of the observed intestinal ulcers.

In mice, rats, and dogs, 1,000 to 10,000 r. of gamma or X radiation suppress mitotic activity in the epithelium of the small intestine to such an extent that cell production cannot keep pace with cell desquamation; hence, the mucosal surface is denuded of epithelium.[1-4] Large volumes of fluid and electrolytes are lost via the gastrointestinal tract, and death occurs within 3 to 5 days after irradiation.[2] The syndrome is referred to as acute intestinal radiation death.[1] The colon is relatively unaffected, and bacteria are said to play a minor role in the process.[5,6]

In monkeys receiving 1,500 to 7,500 r. of gamma radiation, the most severe anatomic alterations at death are in the gastrointestinal tract. The small intestine is not preferentially damaged; the colon is equally or perhaps more severely affected. Gastric and colonic ulceration is frequent. The survival time of 4 to 9 days is considerably longer than in the aforementioned species.[7]

MATERIAL AND METHODS

Ninety-nine monkeys were divided into groups of 5 to 8 animals. The animals weighed from 5 to 7 pounds each and were clinically free of disease. Throughout the experiment they were maintained under conditions described elsewhere.[7,8]

Allen and his colleagues[7] have described the radiation facility utilized. In brief, 32 cobalt^{60} sources rated at 237 curies each were distributed uniformly over a spherical wire frame 36 inches in diameter. The dose rate in the exposure chamber centered in the sphere was 803 r. per minute. There was less than 5 per cent variation in dose rate throughout the exposure field. Radiation doses ranged from 400 to 40,000 r. (Table I).

The animals were fed and watered at 9 a.m. Each animal was irradiated separately and in sequence between 10:45 p.m. and 6:30 a.m. Following exposure, they were kept in individual cages. Every

* Received for publication, April 10, 1959.

animal was subjected to complete necropsy examination immediately after death. Two sets of tissue samples were obtained. One set was fixed in Bouin's solution, and the other was placed in neutral 10 per cent phosphate-buffered formalin. The tissue was paraffin-sectioned

TABLE I

Grouping of Animals for Irradiation

Duration of irradiation	Dose (r.)	No. in group	No. dead in 30 days	Mean survival time (hr.)*
29 sec.	400	5	1	538
37 sec.	500	7	6	359
42 sec.	575	6	4	287
48 sec.	650	6	2	355
54 sec.	725	6	6	302
1 min. 3 sec.	850	4	4	331
1 min. 52 sec.	1,500	8	8	190
2 min. 29 sec.	2,000	8	8	150
3 min. 7 sec.	2,500	8	8	156
4 min. 22 sec.	3,500	8	8	143
6 min. 15 sec.	5,000	8	8	142
9 min. 23 sec.	7,500	5	5	138
12 min. 31 sec.	10,000	5	5	48
18 min. 47 sec.	15,000	5	5	13.6
31 min. 19 sec.	25,000	5	5	6.7
50 min. 7 sec.	40,000	5	5	3.9

* The radiation was administered in a single dose at the rate of 803 r. per minute. Survival times were calculated from the beginning of the irradiation procedure for each animal.

and stained with hematoxylin and eosin. Special stains were utilized when indicated.

A previous report presented the pathologic features in 20 subjects dying a "central nervous system death" (CNS).[9] With two exceptions these were animals irradiated with 10 kiloroentgens or more. One monkey which received 10,000 r. survived beyond the CNS phase; another receiving 5,000 r. died in the CNS phase. Both of these are excluded from the statistical data in this paper, but insofar as the morbid anatomy is concerned, the animal which received 10,000 r. is categorized as an example of acute intestinal death. The remaining animals under consideration in this report are those which received between 1,500 and 7,500 r.

The necropsy specimens from each animal were first examined grossly and microscopically without an exact knowledge of the radiation doses administered to each. The anatomic and clinical diagnoses

usually coincided.[7] Another set of sections was assigned code numbers, the key to which was known only by one technician. The histologic preparations examined in this fashion included the following: 3 sections of stomach, one from the fundus, one from the body, and one from the prepyloric region; 3 sections of small intestine, the duodenum, jejunum, and ileum; and 3 sections of the colon, one from the cecum, one from the sigmoid colon, and one from the rectum. All histologic interpretations were completed before the doses administered were revealed to the examiner.

Results

The 45 monkeys receiving between 1,500 and 7,500 r. exhibited a relatively uniform physical reaction which has been discussed at length by Allen and co-workers.[7] Following the initial episodes of vomiting, diarrhea, nystagmus, and tremors occurring during and immediately after irradiation, the animals rapidly recovered. By the fifth day after irradiation, however, anorexia and diarrhea reappeared and persisted. Emaciation and dehydration were prominent in all of the animals and became more severe the longer the animal survived. In some cases there was as much as a 30 to 35 per cent weight loss by the time of death. The first death occurred on the fourth day and the last on the ninth day. The single animal to die on the fourth day received 5,000 r. On the fifth day, two animals receiving 7,500 r. and one receiving 5,000 r. died. On the sixth, seventh, and eighth days the majority of the animals died, and there was indiscriminate mixing of dose groups with the exception of the 8 animals which had had 1,500 r. Of these, 4 died on day 8, and 4 on day 9. The daily distribution of deaths is illustrated in Text-figure 1. The survival times of individual animals and groups of animals are depicted in Text-figure 2.

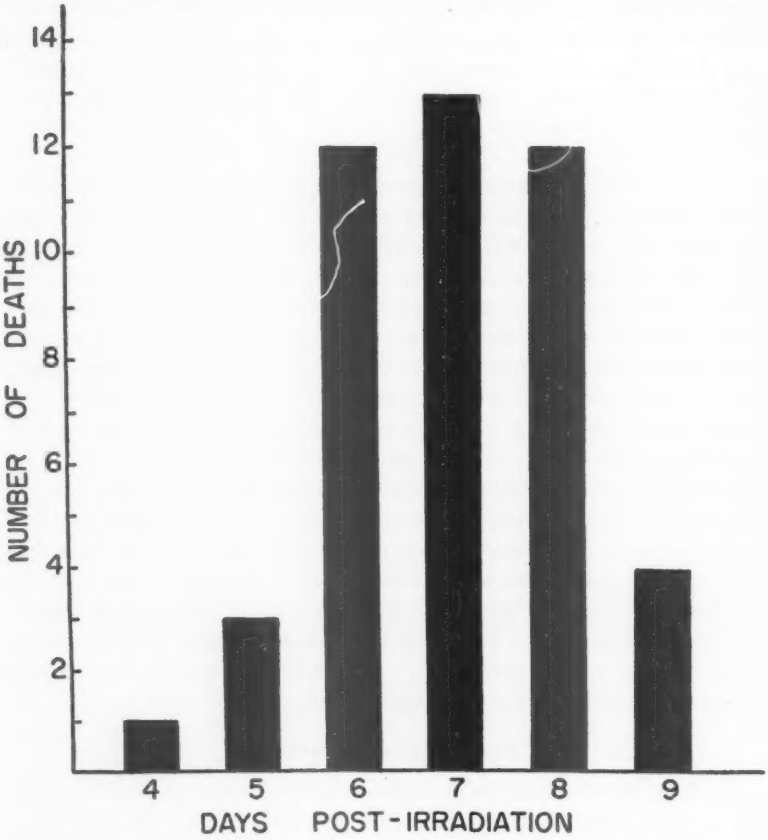
Gastrointestinal Lesions

Mucosal atrophy was consistently noted in the stomach, small intestine, and colon. Although the histologic alterations in the small intestine and colon were essentially the same, the incidence of ulceration in the two organs was strikingly different.

Stomach. In 14 animals the gastric mucosa was ulcerated. Typically, such an affected area was covered by a light green fibrin membrane containing numerous colonies of bacteria. The mucosa was hemorrhagic, but only superficially eroded (Fig. 1). There were occasional faint remnants of gastric glands widely separated by an edematous, hemorrhagic lamina propria (Fig. 2). Bacteria could be found invading the stomach wall, occasionally as far as the subserosal lymphatics. The hemorrhagic necrosis, however, was usually limited to the mucosa.

The muscularis mucosae was generally intact. Immediately beneath the ulcerated region, the submucosa was severely edematous.

The repetitious pattern of gastric ulceration was striking. In each instance, ulceration occurred primarily in the body of the stomach.

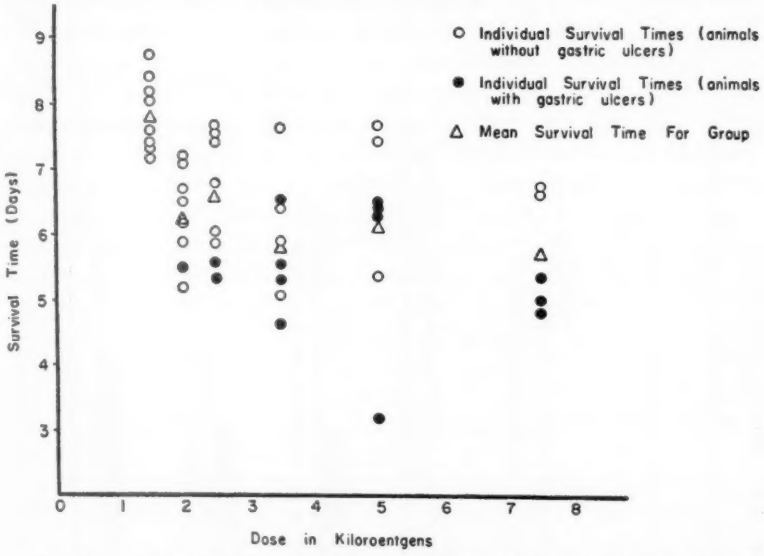


Text-figure 1. Daily distribution of deaths. The animal dying on day 4 received 5 kr. Two animals receiving 7.5 kr. and 5 kr. died on day 5. On days 6, 7, and 8 there was indiscriminate mixing of dose groups except in the animals receiving 1.5 kr. Four of these died on day 8 and 4 died on day 9.

The fundal and prepyloric mucosae were relatively unaffected. Hence, when the stomach was opened along the greater curvature, there was a tendency for the ulcerated areas to assume a butterfly configuration (Fig. 1). It is notable that this is the portion of the mucosa richest in parietal cells.

The incidence of gastric ulceration increased strikingly as the total

dose of radiation increased (Text-fig. 3). This phenomenon was analyzed by the chi square (χ^2) method, although it was necessary to combine adjacent dose groups in order to fulfill the requirements of this test.[10] Thus, the 1.5 and 2.0 kr. groups, the 2.5 kr. and 3.5 kr.

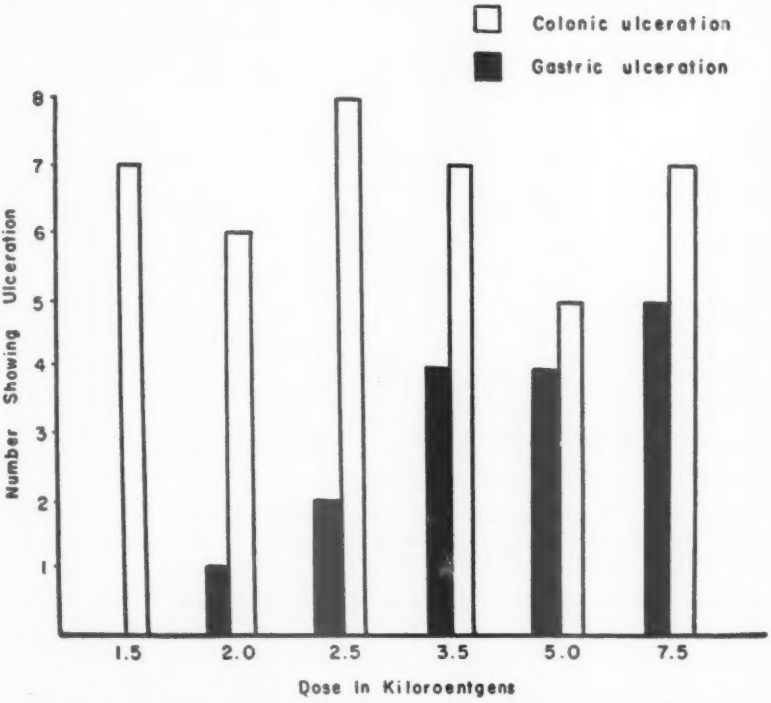


Text-figure 2. Survival time decreased only slightly with increasing dose, particularly in the dose range of 2.0 kr. to 7.5 kr. The survival time of the animals receiving 1.5 kr. was significantly longer than the higher dose groups.

groups, and the 5.0 and 7.5 kr. groups were combined for this consideration. The statistical analysis yielded a χ^2 value of 9.008, which for two degrees of freedom is significant beyond the 2 per cent confidence level and demonstrated that the frequency of ulceration increased significantly as a function of relative radiation dose.

The data in Text-figure 2 suggest that there might be a correlation between gastric ulceration and survival time. In order to determine this relationship independently of dose, the partial correlation technique of Guilford was employed.[11] The point biserial correlation coefficient, a product moment coefficient, for dose and ulcers was computed to be + 0.4706. This value is significant beyond the 1 per cent confidence level, substantiating the positive relationship between dose and occurrence of ulceration. The product moment correlation coefficient for dose and time to death was found to be −0.5739 ($P = 0.01$), demonstrating that the higher the dose, the sooner the animals died. The point biserial correlation coefficient between ulcers and time to

death was −0.6862, giving a negative relationship between occurrence of ulceration and time to death. After ruling out the influence of dosage by using the partial correlation technique, the correlation between ulcers and death was found to be −0.576, a value which is still significant beyond the 1 per cent confidence level. Hence, animals with gastric ulcers died earlier than animals without gastric ulcers, irrespec-



Text-figure 3. The incidence of gastric ulceration by dose groups in comparison with the incidence of colonic ulceration. The incidence of colonic ulceration was invariant with dose. The incidence of gastric ulceration increased with increasing dose. The values for the animals receiving 7.5 kr. are proportional since there were only 5 monkeys in this group.

tive of dose (Text-fig. 2). Extracting the relationship between ulcers and each of the variables, dose and death, the correlation between dose and death was found to be −0.3909 ($P = 0.01$).

In 31 animals the gastric mucosa was grossly intact, but in 19 of these there were varying degrees of atrophic gastritis. Again, the gastric glands of the body of the stomach were the most severely affected (Fig. 3). Occurrence of the more severe instances of mucosal atrophy was random with regard to survival time and dose. However, of the 12 animals which failed to develop atrophic gastritis, 5 received only

1,500 r., 5 received 2,000 r., and there was one animal each from the 3,500 and 7,500 r. groups.

Small Intestine and Colon. Gradations of mucosal atrophy were seen and could be categorized into 4 types, reflecting the severity of cytologic distortion and degrees of epithelial regeneration.

Type I lesions were characterized by severe cytologic distortion of the residual epithelial cells (Fig. 4). The mucosal surface was covered by a thin layer of bizarre cells with many squamoid features. The voluminous cytoplasm was markedly eosinophilic. The cells often assumed a diamond shape and interdigitated with adjacent similar cells. There were broad fields of dense lamina propria widely separating residual intestinal glands, and the glands themselves were lined by a few flattened cells with atypical nuclei. Quite often only the portion of the glands abutting the muscularis mucosae could be identified in the histologic preparations. The bodies of the glands were so atrophic as to merge imperceptibly with the cellular lamina propria. Even though there was a marked reduction in the number of surface epithelial cells, the physical integrity of the mucosal surface was maintained by a continuous thin covering of squamous-like cells. This lesion is referred to as "squamoid atrophy."

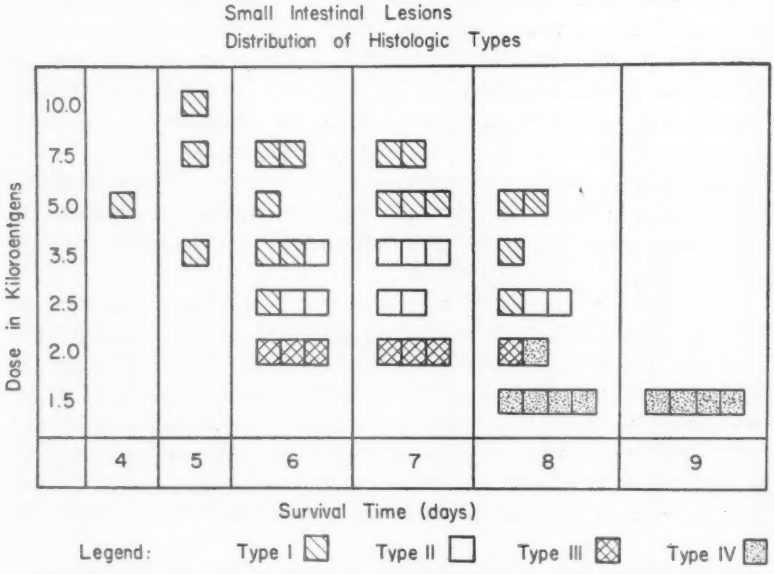
In type II lesions there were also severe cytologic distortion of residual epithelial cells and marked destruction of mucosal glands. However, regenerative efforts were manifested by the proliferation of large, bizarre, cuboidal cells along the surface of the mucosa (Fig. 5). Mitotic figures were frequent in the surface epithelium, but there was very little regenerative activity in the basilar halves of the mucosal glands, the area usually associated with proliferative activity. This type of lesion is designated "squamoid atrophy with surface regeneration."

Type III lesions resembled those of type II in that they also exhibited surface regeneration. In addition, the mucosal glands were markedly atrophic. Here, however, nests of cells retaining many of their normal cytologic features could be found in the surface epithelium (Fig. 6). The nuclei were basilar in position and uniform in size. The shape of the cells was approximately normal, and the cytoplasm was pale pink. Within these nests of cells, the brush borders could be identified. Because of these physical characteristics, this lesion is referred to as "partial atrophy with surface regeneration."

Type IV lesions were among the most interesting found. In their degree of cytologic distortion and glandular atrophy, they were quite similar to the type III lesions. There were, perhaps, slightly larger numbers of apparently normal epithelial cells remaining in the surface

epithelium. The distinctive feature that separated this lesion from the other 3 types was glandular regeneration (Fig. 7). Although the regenerated glands were moderately distorted, and lined by markedly hyperplastic epithelium, they did represent the first attempt at restoration of a relatively normal architecture. This type of lesion is designated "basilar regenerative" and at times was so extensive as to result in complete re-epithelialization of the intestinal mucosa.

Each of the 4 types of lesions was found in the small intestine, and their distribution appeared to depend more on radiation dose than on survival time. By contrast, in the colon the distribution of the lesion



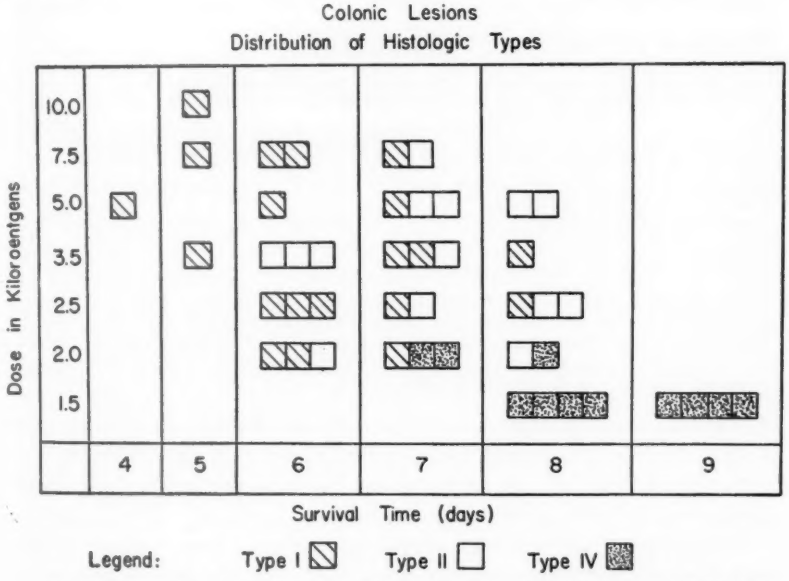
Text-figure 4. There were 4 basic histologic patterns in the small intestine. Their distribution is more closely linked with dose than time. Each pattern's distinguishing characteristics were dependent upon the degree of epithelial regeneration and the severity of cytologic atypism. Each square represents one animal.

types appeared to be more a function of survival time than radiation dose, and type III lesions were not seen (Text-figs. 4 and 5).

There were striking differences in the gross appearances of the small intestine and colon even though the histologic alterations in the 2 organs were basically the same. In only 2 animals was the mucosa of the small intestine ulcerated. Each of these was characterized by mucosal necrosis located over a large submucosal hematoma in the terminal 20 cm. of the ileum. In 4 other animals there were scattered mucosal petechiae. However, the extensive mucosal denudation and

congestion said to characterize intestinal radiation death in other species were not seen in these monkeys, although colonic ulceration occurred in most of them.

As in the stomach, the repetitious pattern of ulceration in the colon was the most striking facet of this entity. Two typical examples are illustrated in Figures 8 and 9. Figure 9 also illustrates a focus of ileal hemorrhage. In Text-figure 6 the incidence of ulceration in the various segments of the colon is depicted. The cecum and ascending colon were far more susceptible to ulceration than the descending colon. Morphologically, the colonic ulceration was similar to that of the stomach.



Text-figure 5. Only 3 of the 4 basic histologic lesions observed in the small intestine were found in the colon, and their distribution (with the exception of type IV) was more a function of survival time than radiation dose. Each square represents one animal.

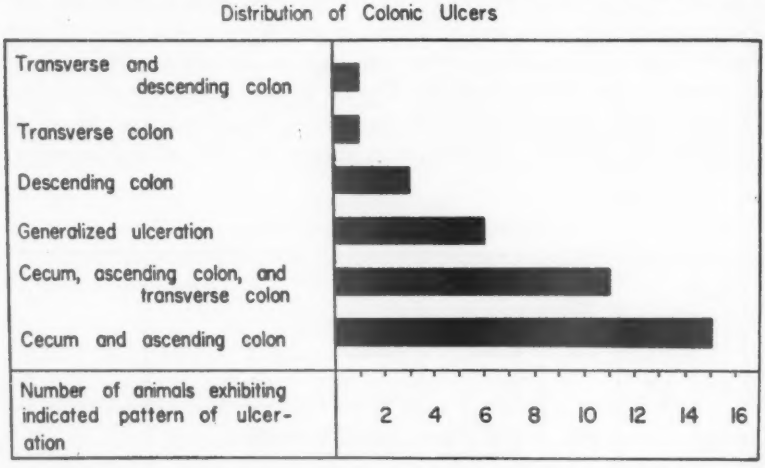
Bacterial invasion of the submucosa and lymphatics was generally more prominent in the colon, and occasionally masses of bacteria were observed infiltrating the lymphatics of the mesentery and pericolonic adipose tissue.

Miscellaneous Observations

In most instances the medullary portions of the kidneys were moderately congested, and in 4 animals there was extensive interstitial hemorrhage in the renal medulla. Twelve animals had renal cloudy swelling. Typically, the involvement consisted of only focal areas of

tubular edema. In 4 animals, however, the edema was generalized, and in 2 of these it was severe.

In the mouth, larynx, and esophagus, the squamous epithelium exhibited moderate nuclear atypism. The nuclei varied in size, and the



Text-figure 6. Each bar of the graph indicates the total number of animals exhibiting the indicated pattern of ulceration. The predisposition for ulceration in the first half of the colon is striking. In only 5 animals was there ulceration of the colon without involvement of the cecum. On the other hand, in 26 animals the ulcerative process involved the cecum without affecting the descending colon.

chromatin was irregularly arranged. Mitotic figures were more numerous than usual. Frequently, colonies of bacteria were adherent to the laryngeal mucosa. In 2 animals the laryngeal mucosa was ulcerated and extensively infiltrated by bacteria.

In approximately one third of the animals the acinar pattern of the pancreas was almost completely obliterated, and there were only rare elements identifiable as acinar cells. Replacing the normal architecture were intertwining masses of poorly differentiated cells. These were probably of acinar origin and had lost their characteristic cytologic features. The acinar pattern was preserved to some extent, as revealed by reticulum stains, but the reticulum was more dense than normal, and delicate strands extended into the centers of the acini.

In all of the animals the bone marrow and lymphoid tissue were severely atrophic.

Although 2,000 r. of gamma radiation are sufficient to induce acute meningitis and vasculitis in the central nervous system,[18] no radiation-induced lesions were found in the brains of these animals. If any had existed, they had resolved by the time the animal died.

The cardiovascular system, the liver, the biliary tree, the skin and the skin appendages were singularly free of lesions. All of the animals considered in this report were prepubertal; no morphologic alterations were observable in the prostate, testis, or uterus. There was early degeneration of scattered oocytes.

Discussion

Monkeys irradiated with acute doses of gamma radiation sufficient to produce death within 3 days fail to develop the atrophic alterations in the intestinal epithelium characteristic of radiation-induced gastrointestinal death.[7,9,12,13] In addition, total body irradiation with 900 r. ± 50 r. is not sufficient to induce the typical cellular changes consistently.[7] Occasionally, an animal irradiated with 900 r. of x-rays from a 250 KV Picker therapy machine exhibits focal epithelial lesions.[14] Optimum radiation doses of 1,500 to 7,500 r. resulted in death of animals between 4 and 9 days. All of the animals developed atrophic enterocolitis which could be divided into 4 types, reflecting the degree of cytologic atypism and epithelial regeneration.

All 4 types of mucosal atrophy were seen in the small intestine, and their distribution was to a great extent dependent upon total radiation dose (Text-fig. 4). On the other hand, only types I, II, and IV lesions were found in the colon. This might have been due to the inherent differences in architecture in the two organs. That is, the cells which were the last to lose their characteristic cytologic features after irradiation ordinarily were the epithelial cells covering the surface of the mucosa. The villi of the small intestine possess far more surface cells than comparable structures in the colon. Hence, there is a better chance numerically of finding normal cells in the former than in the latter. There are reasons, however, to suspect that the absence of type III lesions from the colon may be real rather than artifactual. One such indication in the colon was the fact that the distribution of the various types of lesions was more dependent upon survival time than upon total dose received (Text-fig. 5). Since the various categories reflected to a certain extent the degree of regeneration present, the pattern of distribution within the dose range tested would suggest that the regenerative power of the small intestine was compromised at a lower dose level than that of the colon. Since the various categories were also a measure of cytologic distortion, it may be inferred through similar reasoning that higher total doses of gamma radiation were required to obliterate all normal cytologic detail in the small intestine than in the colon.

Worthy of special comment was the phenomenon of basilar epithelial regeneration observed in the small intestine and colon. In both

organs this lesion was distributed as a function of dose. There was a time correlation, however, since a certain lag period was required for the development of any lesion following the initiating trauma. The dose relationship was emphasized by the fact that every animal receiving 1,500 r. exhibited extensive basilar regeneration. In contrast, only an occasional regenerative gland was found in animals receiving 2,000 r. In addition, on the eighth day after irradiation, 4 animals receiving 1,500 r. died exhibiting basilar regeneration while 7 other animals receiving between 2,000 and 5,000 r. failed to do so (Text-figs. 4 and 5).

In spite of the qualitative similarity in the epithelial lesions in the small intestine and colon, there was a striking discrepancy in the incidence of ulceration in the two organs. Only two ileal ulcers were found, and these appeared as deeply penetrating defects over large submucosal hematomas in the lower 20 cm. of the ileum. On the other hand, colonic ulceration occurred in 33 of the subjects. The incidence of colonic ulceration was invariant over the dose range tested (Text-fig. 3).

Aside from digestive function, one of the most obvious differences between these two segments of the gastrointestinal tract is the bacterial flora. While the small intestine is not completely sterile, the bacterial flora is quite insignificant in comparison to that of the colon.[15] In addition to the marked difference in the incidence of mucosal ulceration in the small intestine and colon, the tremendous predilection of the first half of the colon as compared to the lower half of the colon was interpreted as an indication of the primary role of bacteria in producing ulceration, once the immune mechanism and leukocytes were eliminated by total body radiation.

The role of the abrasive action produced by a passage of the fecal stream has been considered of primary importance by some investigators. This was particularly emphasized by Friedman[16] in a study using colostomies to divert the fecal stream in irradiated rats. He did not, however, completely discount the bacterial factor. In the current investigation the mechanical factors are regarded as secondary. The reasons for these conclusions are threefold. Both the small intestine and colon experience a flow of particulate matter. The small intestinal contents are relatively fluid, but so are the cecal contents. Firm fecal material, which possesses more abrasive properties than liquid feces, is usually not formed until at least the level of the hepatic flexure. Hence, the area of maximum ulceration occurs at the point of maximum stasis of liquid fecal material and not in the area subjected to maximum

abrasive trauma. It has also been shown in some species that the bacterial content of the cecum is about 100 times more concentrated than that of the sigmoid.[15]

It is reasonable to assume that an acute dose of 10,000 r. of gamma radiation would be at least as efficient as 7,500 r. in producing gastric ulceration. It has been observed, however, that animals irradiated with 10,000 r. or more die within 3 days and fail to develop ulcers.[9,12,18] Therefore, it is concluded that at least 4 days are required for the development of such lesions. After 4 days, the incidence of ulceration is a function of dose rather than time. As the total dose is increased, the incidence of gastric ulceration increases. The finding of a significant negative correlation between ulcers and time of death (−0.576), with the influence of dosage removed, is interpreted as a negative value, meaning that those animals which develop ulcers die sooner than those animals which do not, irrespective of dosage. This observation assumes greater significance when it is noted that the incidence of ulceration increases as the radiation dose is increased. Under these conditions, ulceration becomes an important factor in determining the slope of the "dosage versus time" mortality curve (Text-fig. 2). Radiation dose nevertheless remains a determining factor, as attested by the lower significant correlation (−0.3909) between dose and death, when the relation of each with ulcers is extracted.

Since gastric ulceration was limited to that portion of the mucosa richest in parietal cells, it appeared probable that selective cell damage was an important factor in regulating the repetitious pattern of the lesions. The predilection of this particular cell for radiation damage was emphasized by the pattern of mucosal atrophy as found in this experiment, as well as the experiments of others.[7,9,12,13] However, there are two other facets to the problem which cannot be disregarded. First of all, although in their most severe form the ulcers assumed a butterfly configuration, the smaller lesions were limited to the mucosa of the greater curvature, the most dependent portion of the stomach. Secondly, among apparently normal animals sacrificed at random in this laboratory, bacteria were not infrequently found in the lumens of the gastric glands richest in parietal cells. Hence, the same principles applicable to the pathogenesis of colonic ulcers are thought also to affect the development of gastric ulcers under the conditions of this experiment. That is, intrinsic bacterial flora were able to penetrate the damaged mucosa and produce tissue necrosis and hemorrhage. The particulate matter contributed to ulcer production because its presence favored bacterial growth and less because it tended to scrape cells off the mucosa as it passed along the gastrointestinal tract.

Summary

Forty-four monkeys irradiated with 1,500 r. to 7,500 r. of cobalt^{60} (gamma) radiation died between 4 and 9 days after irradiation. The greatest percentage of deaths occurred on the sixth, seventh, and eighth days. The mean survival time was 6 days. The most prominent findings at necropsy were gastric and colonic ulceration. Colonic ulceration occurred in 33 of the animals, and its incidence was invariant with radiation dosage over the dose range tested. Gastric ulceration occurred only 13 times. Its incidence increased as radiation dose increased. This observation was analyzed statistically. Evidence presented suggests that bacteria play a dominant role in the production of gastric and colonic ulceration following supralethal doses of radiation.

Severe mucosal atrophy occurred in both the small intestine and colon. It could be divided into 4 basic types which reflected the severity of cytologic distortion of the epithelial cells, and the degree of epithelial regeneration. In the small intestine, the distribution of the lesion types was a function of radiation dose; in the colon the distribution was a function of survival time. The significance of these variations is discussed.

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All statistical analyses were performed by Dr. A. A. McDowell, Department of Psychology, University of Texas.

[*Illustrations follow*]

LEGENDS FOR FIGURES

Except as indicated, histologic preparations photographed were stained with hematoxylin and eosin.

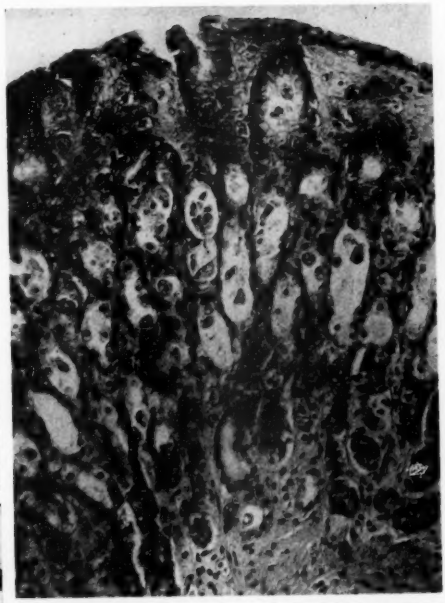
FIG. 1. Stomach of animal dying 6 days after irradiation with 5 kr. The hemorrhagic necrosis is limited to the parietal cell-containing region of the gastric mucosa. A thin fibrin membrane covers portions of the necrotic mucosa.

FIG. 2. Photomicrograph of the gastric mucosa illustrated in Figure 3. The lamina propria is hemorrhagic and contains only remnants of gastric glands. There are distorted remnants of gastric glands in the basilar areas. × 250.

FIG. 3. One of the more severe cases of atrophic gastritis. There are desquamated parietal cells in the glandular lumens. The basilar portions of the glands are mildly atrophic, but the superficial epithelium appears normal. Animal receiving 5 kr. × 130.

FIG. 4. Small intestine of animal dying 5 days after irradiation with 3.5 kr. Only degenerating, bizarre, squamous-like cells remain on the mucosal surface. There is severe atrophy of the glands; type I lesion. × 200.

1



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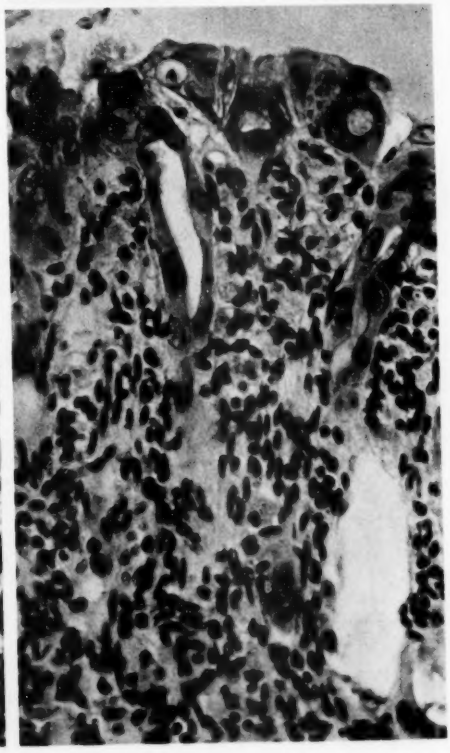

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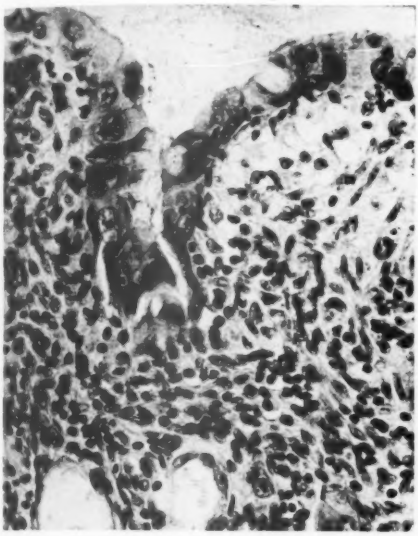
FIG. 5. "Columnar regeneration" along the surface of the colonic mucosa. This is a type II lesion. Note that the glands are still markedly atrophic. There were occasional mitotic figures in the bizarre epithelium along the mucosal surface. From animal dying 6 days after irradiation with 3.5 kr. × 150.

FIG. 6. A type III lesion (ileum) from animal dying 7 days after irradiation with 2.5 kr. The epithelial cells are atypical, but still retain some of their normal characteristics. × 200.

FIG. 7. A type IV lesion (colon) from an animal dying 8 days after irradiation with 1.5 kr. Residual atrophic glands remain, but note the regenerating glandular structures lined by markedly hyperplastic epithelium. There is residual degenerating epithelium along the mucosal surface, but the regenerating epithelium exhibits creeping replacement. × 200.

FIG. 8. Cecum and ascending colon of animal dying 5 days after irradiation with 7.5 kr. The edematous mucosa contains extensive foci of hemorrhage. The dark areas are hemorrhagic. The light areas are covered by fibrin membranes.

FIG. 9. An example of the ulcerative colitis. Note that the first half of the colon is affected, but that the distal half appears grossly normal. Also illustrated is one of the two localized areas of ileal hemorrhage observed in the 45 animals. From monkey dying 7 days after irradiation with 2 kr.



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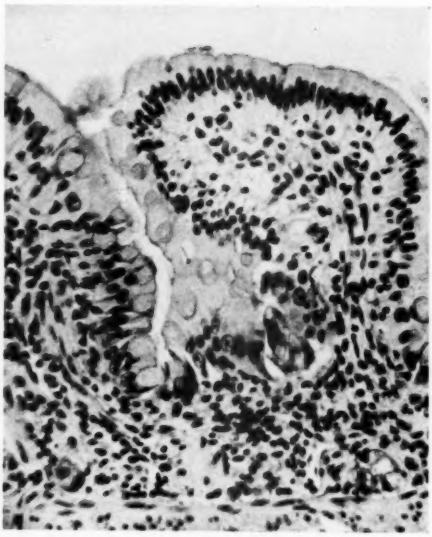

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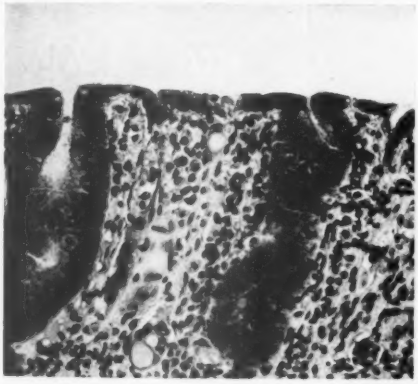

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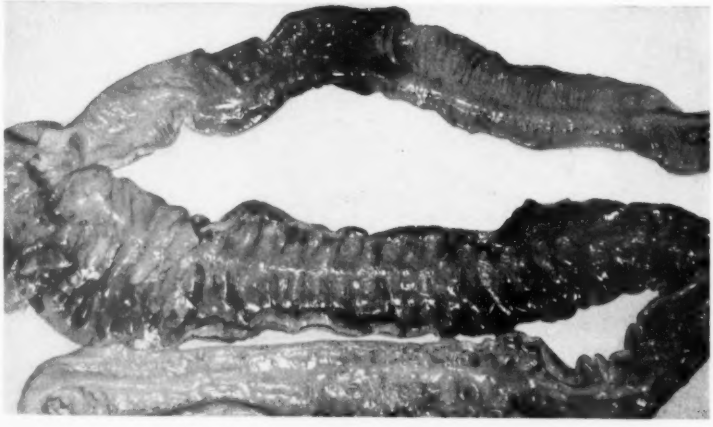

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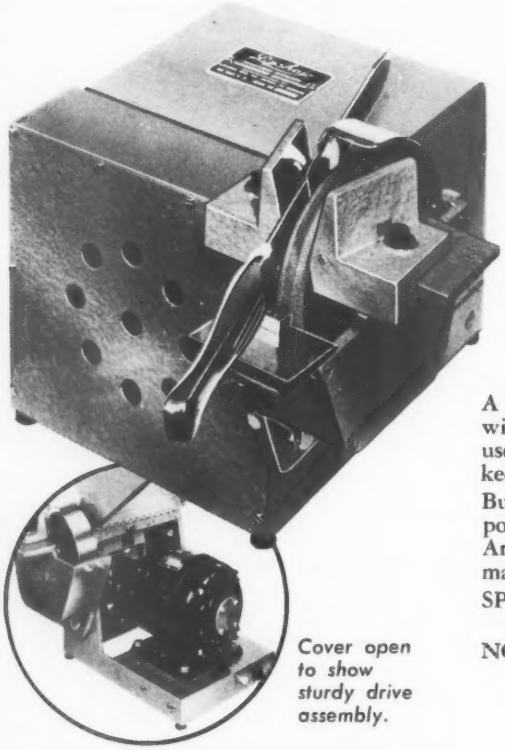

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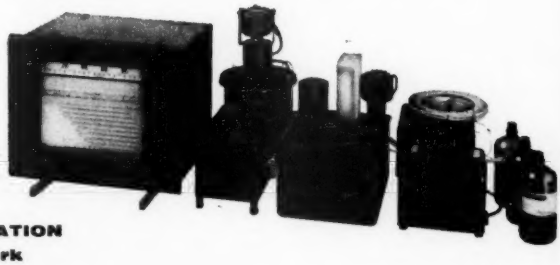

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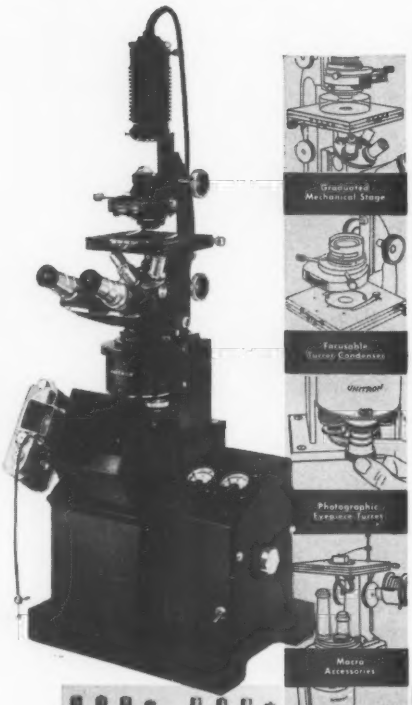

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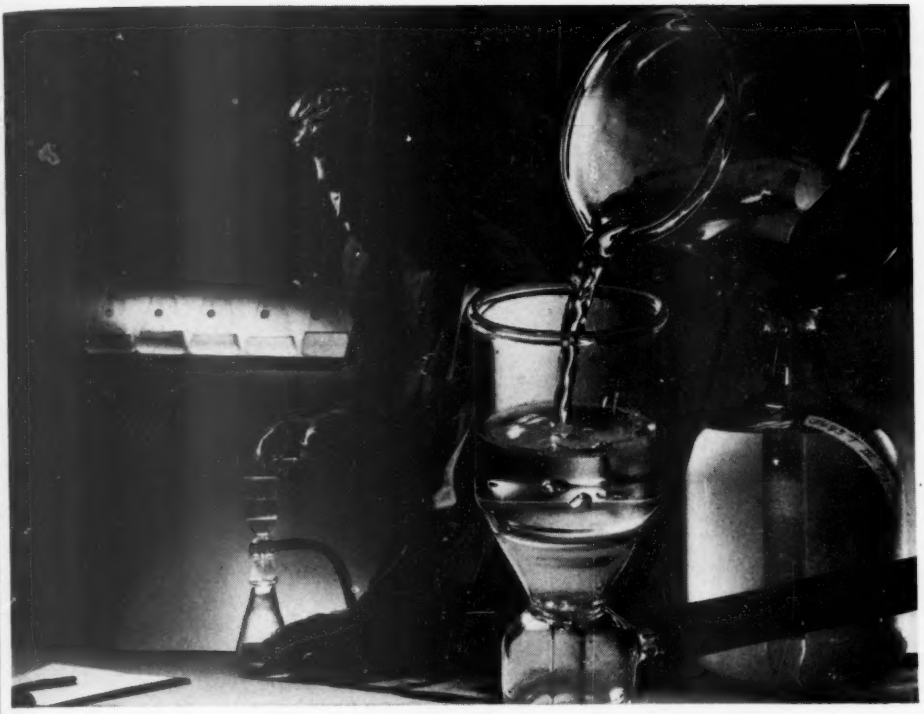

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